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# COMPARATIVE BIOCHEMISTRY & METABOLISM: PART II NAPHTHALENE LUNG TOXICITY

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## TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

**FOR THE COMMANDER**

BRUCE O. STUART, PhD
Director Toxic Hazards Division
Air Force Aerospace Medical Research Laboratory

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Earlier studies have shown that the volatile aromatic hydrocarbon, naphthalene, produces a highly selective lesion of the pulmonary bronchiolar epithelium in mice. This lesion appears to depend upon the cytochrome P450-mediated metabolism of the parent compound, and glutathione is important in modulating the cytotoxicity. Although an excellent correlation was noted between the formation and covalent binding of highly reactive naphthalene metabolites in the target tissue with the extent and severity of the pulmonary lesion, additional studies suggested that the levels of covalently bound metabolite found in a particular tissue in vivo did not necessarily reflect the rate at which reactive metabolites were formed in situ within that tissue. Indeed, studies conducted during the current contract year have demonstrated that naphthalene oxide formed intracellularly is capable of effluxing from intact hepatocytes.

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In the presence of microsomes, glutathione and the glutathione transferases, naphthalene is metabolized to at least three glutathione adducts which can be separated and quantitated by high pressure liquid chromatography. Since previous work indicated that the rates of formation of the three adducts differed markedly in microsomes from target vs nontarget tissues, the thrust of the studies done in the last contract year was to identify the three adducts and to examine the kinetics of formation of each of these derivatives in target and nontarget tissues from sensitive and nonsensitive species.

Fast atom bombardment mass spectrometry and synthesis of all three adducts from (±)-naphthalene oxide indicated that the adducts formed are most likely positional and enantiomeric forms of hydroxy-dihydroglutathionylnaphthalene. Kinetic studies in target and nontarget tissue microsomes indicated major differences in the $V_{max}$ but not the $K_m$ for the formation of naphthalene glutathione adducts.

The metabolism of naphthalene by human lung microsomes occurred at easily detectable rates with the formation of naphthalene dihydrodiol predominating. The rates of metabolism were lower than in rodent lung and there were marked interindividual differences.

Pulmonary microsome-catalyzed formation of 1-naphthol from naphthalene occurs at 2.5 times the rate of 1-naphthol formation in liver microsomal incubations, yet the rate of formation of covalently bound metabolites is similar. This, combined with the demonstration that mouse lung and liver microsomes form covalently bound metabolites from 1-naphthol at nearly identical rates, supports the view that not all of the metabolites which become bound covalently arise through the intermediate formation of 1-naphthol.

In sum, while an excellent correlation between the target tissue specificity for the formation of naphthalene glutathione adduct 2 and the target for naphthalene-induced cytotoxicity has been demonstrated, additional studies will be needed to support the view that the precursor of adduct 2 is important in the toxicity of this aromatic hydrocarbon.

## PREFACE

This is the annual report of the Subprogram in Biochemistry and Metabolism, Part II: Naphthalene Lung Toxicity and concerns work performed by the Department of Community and Environmental Medicine, University of California, Irvine, on behalf of the Air Force under Contract #F33615-80-C-0512, Work Unit 63020115. This document describes the accomplishments of the subprogram from June 1983 through June 1984.

A.R. Buckpitt served as coordinator of the subprogram. Much of the work described in this document was carried out by Ms. Linda Bahnson and her technical expertise in these studies is gratefully acknowledged. The work on naphthalene metabolism by isolated hepatocytes was done by Mr. Paul Richieri and his efforts in designing and executing these studies have been essential to the conduct of this project. Finally, we would like to express our appreciation to Dr. Neal Castagnoli, Jr. and Dr. Sidney D. Nelson for their willingness to collaborate in the identification of the naphthalene glutathione adducts.

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## INTRODUCTION

This report summarizes the work conducted from June 1983 through June 1984 in the Subprogram on Comparative Biochemistry and Metabolism, Part II: Naphthalene Lung Toxicity. The intent of the work conducted in this subprogram is to provide a better understanding of the critical molecular and biochemical events associated with the highly selective pulmonary bronchiolar necrosis observed in naphthalene treated mice. The basic rationale and the importance of these toxicologic investigations to the Air Force and Navy will be discussed briefly in the following paragraphs.

Naphthalene is a widespread environmental contaminant to which humans are exposed. Exposures occur primarily from cigarette smoke (Schmeltz et al., 1976; 1978), fuels and lubricants (Fruchter et al., 1978), diesel particulates (Clark et al., 1982), and in the chemical industry (Gammage et al., 1978; Priority Toxic Pollutants, 1980). Naphthalene is a synthetic starting material in the production of decalin and is present in relatively large quantities in a solvent for ferrocene, a fuel additive currently being considered for use by the USAF/USN (McEwen and Vernot, 1981). Administration of naphthalene to mice by either the intraperitoneal or inhalation routes results in necrosis of the nonciliated bronchiolar epithelial cells. This toxicity appears to depend upon the cytochrome P450-mediated metabolism of the parent hydrocarbon, and tissue reduced glutathione has been shown to be important in modulating lung injury (Warren et al., 1982). Since the bioactivation of chemically inert agents may play a role in lung diseases including fibrosis, emphysema, and cancer (Boyd, 1980; Brody et al., 1981), it is important to understand the underlying molecular and biochemical basis for chemical-induced damage to the bronchiolar epithelium in animals and to extend these studies to the human.

The problem of extrapolation of data from animals to man is especially troublesome when considering those chemicals which produce bronchiolar epithelial cell necrosis in animals. The mouse is substantially more sensitive than the rat to bronchiolar injury by a number of chemicals which appear to require metabolic activation to produce their toxicity. These agents include carbon tetrachloride (Boyd et al., 1980), dichloroethylene (Fokert and Reynolds, 1982; Krijsheld et al., 1983; Chieco et al., 1981), and naphthalene (Buckpitt, 1981). Detailed studies attempting to elucidate the underlying reasons for the species differences in the bronchiolar injury induced by these chemicals have not been reported nor have studies on human lung microsomal metabolism of these chemicals via toxicologically relevant pathways been done. Thus, the aim of studies supported by the U.S. Air Force and U.S. Navy has been to examine differences in the metabolism of naphthalene in target and nontarget tissues as a means to elucidate factors which may play a critical role in the cytotoxicity of naphthalene in rodents and to extend these studies to include metabolism of this chemical by human lung tissue. This report describes studies conducted in the past year which indicate that the nature and rates of formation of reactive naphthalene metabolites (trapped as glutathione adducts) are markedly different in target as compared with nontarget tissues of the rodent.

## BACKGROUND

### Relationship of Metabolism to Naphthalene-Induced Pulmonary Bronchiolar Injury

Reid et al. (1973) were the first to report the pulmonary bronchiolar necrosis in mice after intraperitoneal administration of aromatic hydrocarbons including naphthalene. Subsequent studies have confirmed and extended these findings to show that inhibition of the cytochrome P450 monooxygenases by prior treatment with piperonyl butoxide or depleting tissue glutathione stores by diethylmaleate markedly inhibits or exacerbates, respectively, the pulmonary lesion by naphthalene. Further studies on the possible relationship between the formation and fate of highly reactive metabolites of naphthalene and the pulmonary bronchiolar damage have shown that alterations in the severity of the pulmonary lesion are reflected by parallel alterations in the

level of covalently bound metabolites in the target organ (Warren et al., 1982). However, studies on the relationship between cytotoxicity and covalent binding levels in the lung were complicated by the data showing that reactive metabolites of naphthalene appear to be sufficiently stable to circulate from the liver (Buckpitt and Warren, 1983). Thus, measurements of the levels of covalent binding to tissue macromolecules in vivo may not provide an accurate quantitative estimation of the reactive metabolites formed in situ. There were two possibilities which were still consistent with the concept that the formation and covalent binding of reactive metabolites to critical cell macromolecules was an essential step in the pulmonary lesion induced by administration of high doses of naphthalene. The first was that the sites to which reactive metabolites became bound were different in the target and nontarget tissue, and the second was that the nature and kinetics of reactive metabolite formation differed. The second possibility has been explored in detail and the results of these studies are presented in this report. In addition, further direct evidence that naphthalene oxide is capable of effluxing from intact hepatocytes will be discussed in this report.

**Naphthalene Metabolism**

The metabolism of naphthalene to various oxygenated metabolites has been studied extensively (Jerina et al., 1970; Jerina and Daly, 1974). More recent work has demonstrated the presence of numerous oxygen and sulfur-containing metabolites of naphthalene in the urine of mice and rats (Horning et al., 1980; Stillwell et al., 1982). A total of seven sulfur metabolites, accounting for approximately 39% of the administered dose (100 mg/kg), were identified in mouse urine. Of this, 97% was tentatively identified as N-acetyl-S-(1-hydroxy-1,2-dihydro-2-naphthalenyl)-L-cysteine. This identification was based on mass spectra which were consistent with this assignment and upon previous studies by Jeffrey and Jerina (1975). Our recent studies showing that at least 3 glutathione adducts can be isolated from microsomal incubations of naphthalene, glutathione, and NADPH or from chemical reaction of naphthalene-1,2-oxide with glutathione indicate that the peak isolated by gas chromatography may have contained more than one form of the mercapturic acid derivatives. Van Bladeren et al. (1983) have recently reported the isolation of isomeric mercapturic acids after incubation of naphthalene with purified cytochrome P450 b and P450 c.

## RESEARCH PROGRAM

**General Methods**

Animals

All animals were males and were purchased from Charles River Breeding Laboratories, Wilmington, MA. Mice were Swiss Webster weighing 20-30 g. Hamsters were Golden Syrian (70-90 g), and rats were Sprague Dawley (125-175 g). All animals were held in the animal resource facility at least 5 days after receipt from the supplier and were housed over hardwood bedding in a HEPA/carbon filtered laminar flow cage rack. Animals were given food and water ad libitum.

Radiochemicals

$^{14}$C-Naphthalene was purchased from either Amersham Corporation, Arlington Heights, IL or from California Bionuclear, Sun Valley, CA. Radiochemical purity was checked by HPLC on a $C_{18}$ column using 70% methanol:30% water. All shipments from Amersham were found to be 99.5% radiochemically pure. The first shipment from California Bionuclear was 70% pure and was returned. The product finally supplied from this vendor was > 99.5% purity. L-Glutathione (glycine-2-$^{3}$H ) (reduced form, 240 mCi/mole) was purchased from New England Nuclear, Boston, MA and was used without further purification.

## Chemicals

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, epoxy activated Sepharose 6B, and collagenase were purchased from Sigma Chemical Co., St. Louis, MO. Reduced glutathione was from Calbiochem, La Jolla, CA. Cyclohexene oxide, 1,2-dihydronaphthalene, and other reagents used in the synthetic preparation of naphthalene-1,2-oxide were bought from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were purchased from commercial suppliers and were reagent grade or better.

## Preparation of Microsomal Enzymes

Pulmonary, hepatic, and renal microsomes were prepared by differential ultracentrifugation as described previously (Buckpitt and Boyd, 1980). The supernatant from the first 100,000 x g centrifugation of mouse liver postmitochondrial supernatant was saved for the preparation of semi-purified glutathione transferases. Microsomal and cytosolic protein concentrations were determined by the Coomassie blue dye binding procedure of Bradford (1976).

## Preparation of Semi-Purified Glutathione Transferases

The cytosolic enzymes prepared from mouse liver as described above were chromatographed on a Sephadex G-25 column to remove glutathione as previously described (Rollins and Buckpitt, 1979). One to 3 mL of the glutathione-free supernatant was applied to a 1 x 6.5 cm column containing glutathione agarose. The column was rinsed with 7 mL 25 mM sodium phosphate buffer, pH 7.0, and the glutathione transferases were eluted from the column with 15 mL Tris buffer (50 mM) containing 5 mM glutathione at a pH of 9.6. This fraction was concentrated by pressure dialysis in an Amicon stirred cell (using a PM-10 membrane), and glycerol and glutathione were added to bring the solution to 1% glycerol and 5 mM glutathione. Enzyme was stored at -80$^{o}$C until use. Glycerol and glutathione were removed from the enzyme preparation by dialysis before use. Enzyme activity was assayed with 1-chloro-2,4-dinitrobenzene according to the method of Habig et al. (1974).

## Incubations

Incubations were prepared on ice in a total volume of 2 mL. The incubation vessels contained: microsomal protein (1-6 mg as specified), NADPH-generating system (Buckpitt and Boyd, 1980), glutathione transferases (as specified), reduced glutathione, and $^{14}$C-naphthalene (concentration and specific activity as specified) added in 10 $\mu$L methanol. The incubation vessels were capped, transferred to a shaking incubator at 37$^{o}$C for the specified time period, and then placed on ice. Four mL of ice cold spectral grade methanol was added to quench the reaction, and the contents of the incubation vessel were transferred to a centrifuge tube for HPLC analysis of the polar naphthalene metabolites.

## Analysis of Naphthalene Dihydrodiol and Naphthalene Glutathione Adducts

The samples prepared as described were centrifuged at 8,000 x g for 20 min to remove protein from the sample. The aqueous supernatant was transferred to another centrifuge tube, and unchanged naphthalene was removed by extraction with trimethylpentane (Smart and Buckpitt, 1983). A 1-2 mL aliquot of the aqueous phase was then evaporated under vacuum and reconstituted in mobile phase for HPLC analysis. Chromatography was performed either on a Beckman Ultrasphere ODS column (5 $\mu$m, highly end-capped, spherical $C_{18}$, 0.46 x 15 cm) or on a Waters (Milford, MA) Radial compression module fitted with a Novapak column (5 $\mu$, $C_{18}$, spherical packing that is highly end-capped, 0.8 x 10 cm). The mobile phase consisted of 4-5% acetonitrile/1% glacial acetic acid/94-95% water adjusted to yield complete separation of the

adducts and naphthalene-1,2-dihydrodiol. Metabolites were detected by UV absorbance at 254 nm. These were quantitated by collecting the compounds eluting from the HPLC column directly into scintillation vials. Beckman EP scintillation fluid was added and each sample was counted for 20 min on a Beckman 3150 T liquid scintillation counter.

## RESULTS

### Identification of Naphthalene Glutathione Adducts

#### Purification for NMR and Mass Spectral Analysis

Approximately 1 mg of naphthalene glutathione adducts 1 and 2 were sent to Dr. Neal Castagnoli, University of California, San Francisco for FABMS and NMR. These conjugates were purified from an extract of a 300 mL mouse liver microsomal (PB-induced) incubation containing 1 mM naphthalene, 5 mM glutathione, 3 mg/mL microsomes, cytosol (0.5 mg/mL) and cofactor. The extract from the incubation was chromatographed on an XAD-2 column to remove water-soluble contaminants from the microsomal incubation. The methanol eluate which contained the glutathione conjugates was evaporated, and the naphthalene glutathione conjugates were separated on a Beckman Ultrasphere 5 μ (1.0 x 25 cm) column eluted with 6-8% acetonitrile/1% acetic acid/water at 3 mL/min. Approximately 500 mL of the column eluate containing the separated conjugates was evaporated to dryness under vacuum. The residue was reconstituted in 2 mL water and each conjugate was rechromatographed on the semi-preparative HPLC column. This step removed many UV absorbing contaminants which eluted in the first 10 min of the chromatographic run. (These were most likely due to nonvolatile constituents in the HPLC solvents.) The conjugates from the final step in the purification were collected in glass scintillation vials (previously rinsed in spectral solvents of increasing polarity to remove phthalates) and a small quantity of the collected material was checked for purity on a Beckman analytical column. During the final purification step adduct 3 was lost. Based on the elution of UV absorbing compound, peaks 1 and 2 were judged to be greater than 99% pure. A further aliquot of the purified conjugate was added to an extract from a microsomal incubation to exclude the possibility that evaporation of the conjugates had induced changes in this chemical structure. Naphthalene glutathione adduct 1 and adduct 2 co-chromatographed with adduct 1 and 2, respectively, from the extract of the microsomal incubation.

Sufficient material was present in samples of conjugate 1 and 2 to obtain NMR spectra (data not shown). However, a series of signals in the range of 3.50-3.85 ppm indicated the presence of a major contaminant in our samples. Moreover, the signals normally observed in the NMR of GSH adducts (Moss et al., 1983; Mulder et al., 1982) in the region of 2.0-4.0 ppm were weak. The spectra were consistent with the presence of GSH adducts but could not be interpreted properly to give a more definitive identification of the material. Fast atom bombardment mass spectra of the two adduct peaks (data not shown) again indicated that the compounds sent to Dr. Castagnoli had degraded. Interpretation of the spectra was delayed pending our determination of the purity of the materials by HPLC.

Professor Castagnoli returned the naphthalene-glutathione adducts in January, and each sample was redissolved in 200 μL of water and a 1 μL aliquot was chromatographed on a Beckman $C_{18}$-5 μ column with 5% acetonitrile/1% acetic acid/water. Chromatography of either Peak 1 or Peak 2 indicated the presence of considerable amounts of UV absorbing material which eluted in the column void volume. A UV absorbing peak coeluting with the dihydrodiol was also present in both samples. From the UV absorbance at 254 nm, it was estimated that the respective glutathione adducts constituted only about 50% of the total material in the sample. This does indicate that the compounds are not stable indefinitely.

Synthesis of Racemic Naphthalene Oxide and Demonstration that All Three Glutathione Adducts Are Formed Upon Chemical Reaction with Glutathione

The synthesis of racemic naphthalene oxide has been described in detail by Yagi and Jerina (1975). 1,2-Dihydronaphthalene (Aldrich) (19.3g) was treated with bromine to form 1,2-dibromonaphthalene MP 68°C, 26.3g, 63% yield. 1,2-Dibromonaphthalene [29 g - (3g from previous synthesis)] was refluxed with $MgCO_3$ in acetone to yield 10.5 g 1-hydroxy-2-bromotetralin (45% yield MP 110°-112°C). Ten grams 1-hydroxy-2-1-bromotetralin was acetylated with acetic anhydride to yield 1-acetoxy-2-bromotetralin MP 94-95°C, yield 76.9%. Three grams of the acetoxy bromotetralin was heated with a sun lamp in the presence of 1.6 g N-bromosuccinimide and 5 mg $\alpha$ $\alpha$ -azoisobutyryl dinitrile to yield 1.58 g 1-acetoxy-2,4-dibromotetralin (41% yield). This material was very difficult to recrystallize and the final crystalline product was probably impure (MP 92-96°C). Because naphthalene oxide is very labile, the final step in its synthesis has been done in small batches. The oxide decomposes fairly rapidly at -20°C in dry ethyl ether (approximately 20% decomposition has been noted in 2 weeks). All of the synthetic material has been purified by TLC (silica gel GF, benzene/chloroform/ethyl acetate/triethylamine 1:1:1:0.05) prior to use. The identity of the material was based on ultraviolet absorption spectra ($\lambda$ max 264 and 302 in trimethylpentane) and NMR (Varian FT 80 in $CDCl_3$ with 0.1% TMS as reference) which were consistent with that reported previously (Vogel and Klarner, 1968). Moreover, addition of acetic acid and methanol to the dried material results in complete rearrangement to 1-naphthol.

To determine whether all three glutathione adducts formed during the incubation of lung or liver microsomes with glutathione and transferase enzymes could be generated from the intermediate naphthalene oxide, flasks containing 0.5 or 0.05 mM naphthalene oxide, $^3$H-glutathione (5.0 mM, 82 dpm/nmole), and glutathione transferases were incubated for 30 min at 37°C. The data in Table 1 indicate that all three peaks were formed during such incubations and that the ratio of adducts formed was 1:2:1 at 0.5 mM naphthalene oxide but was approximately 1:1:1 at 0.05 mM. The data also indicate that the nonenzymatic rate of conjugate formation at pH 8.0 was nearly as high as the rate of transferase-mediated conjugation. These data, combined with the results of previous studies with arene oxide glutathione conjugates (Hernandez et al., 1980; Van Bladeren et al., 1982), suggest that the structures outlined below (Figure 1) are likely. There are four possible adducts formed during the reaction of racemic naphthalene oxide with glutathione which raises the possibility that one of the conjugates is either not formed or is not separated using our HPLC conditions. Further attempts have been made to separate a fourth metabolite from an extract of a liver microsomal incubation containing $^{14}$C-naphthalene, glutathione, NADPH, and glutathione transferases. Two 5 $\mu$ $C_{18}$ columns were attached in series, and the acetonitrile concentration in the mobile phase was decreased to 4% to provide good retention of the GSH adducts. The UV profile in Figure 2 indicates that under chromatographic conditions in which the adducts were retained for 60-90 min and which yielded peak to peak separations of at least 6.5 min, a shoulder could not be detected in the UV profile. Moreover, evidence of a shoulder was not obtained when samples of the column eluate were collected at 30 sec intervals for the determination of radioactivity (data not shown). Thus, either the fourth adduct is not formed in sufficient amounts to be detectable [and VanBladeren et al. (1982) have demonstrated that some of possible glutathione conjugate isomers are not formed with benzanthracene] or the chromatographic system we are using is not capable of separating them.

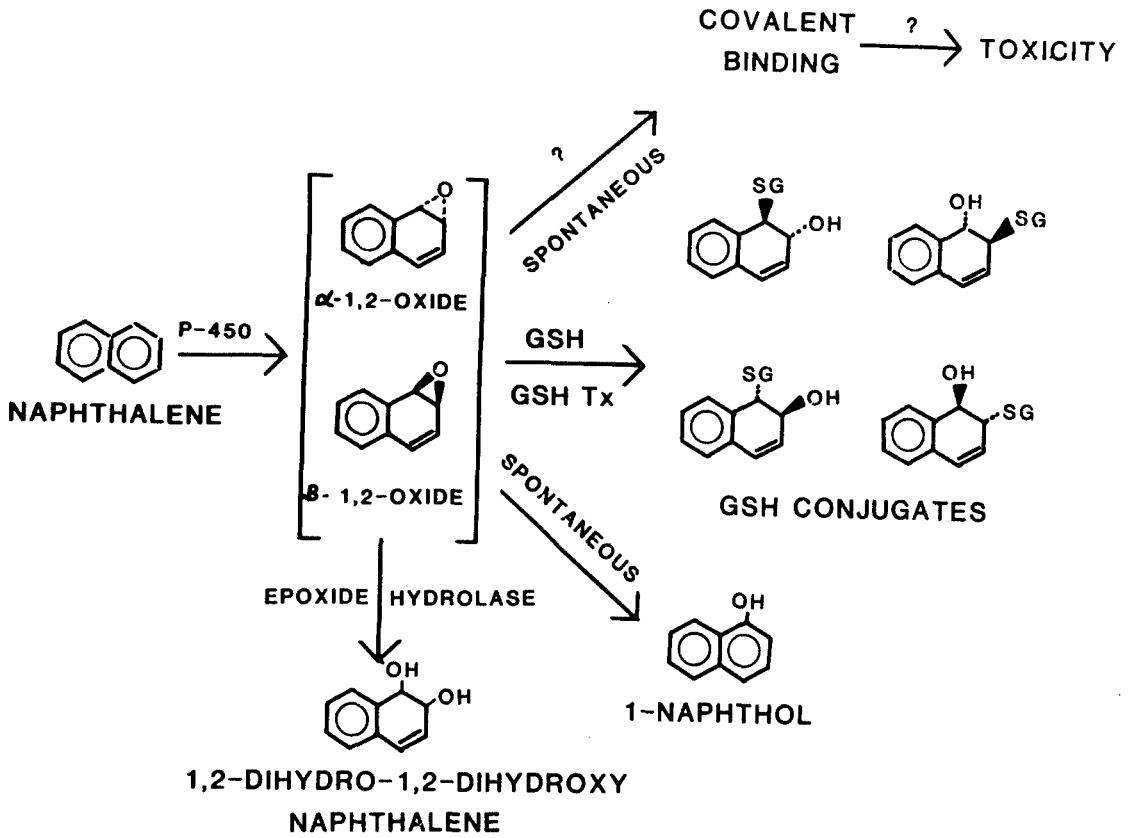



Figure 1. Metabolism of Naphthalene

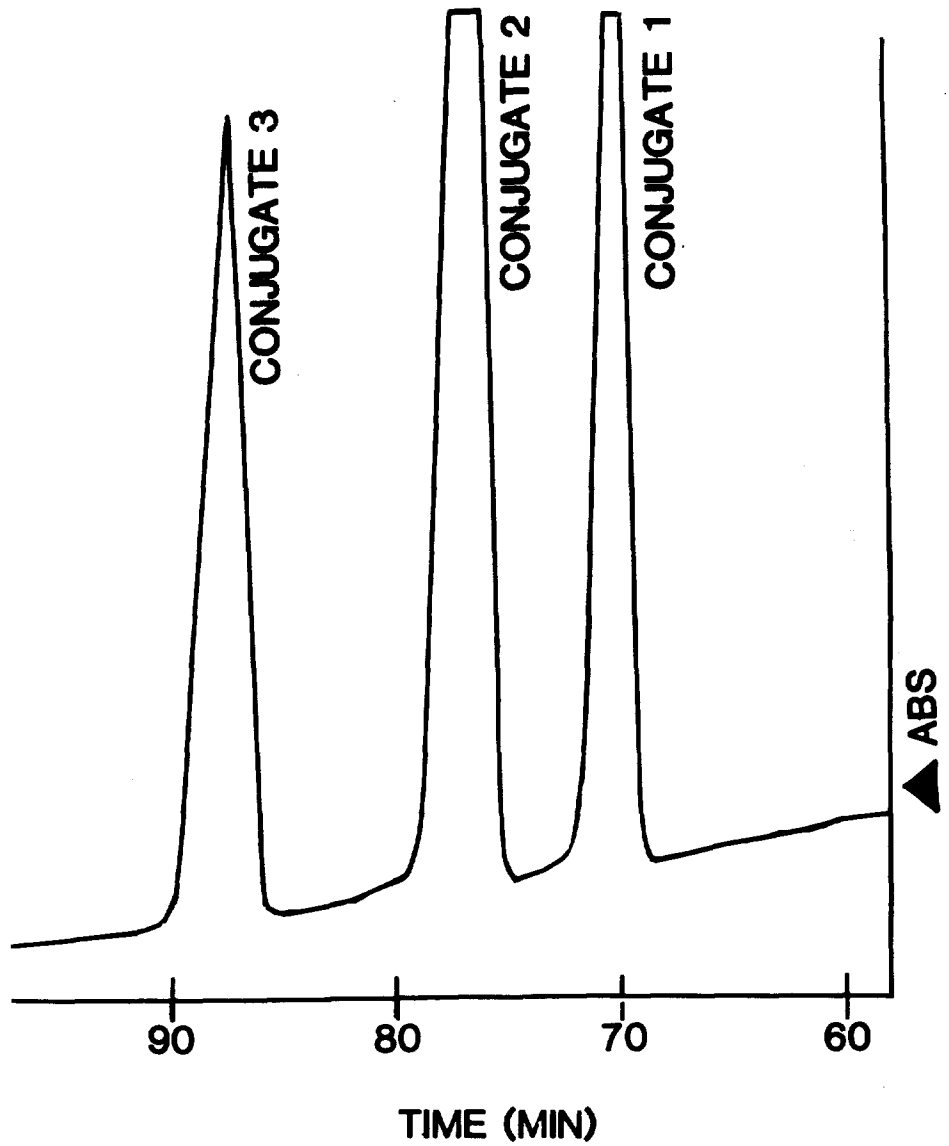



Figure 2. UV HPLC profile of naphthalene glutathione adducts 1,2, and 3 chromatographed using two Beckman $C_{18}$ 5 μ columns attached in series. Chromatographic conditions were: 4% acetonitrile/1% acetic acid/95% water at a flow rate of 1 mL/min.

Table 1

Rates of Formation of $^{3}$H-Labelled Glutathione Adducts in Incubations of Naphthalene Oxide and $^{3}$H-Glutathione Done in the Presence or Absence of the Glutathione Transferases[a]

| Incubation Conditions | Naphthalene oxide | GSH Tx | Conj 1 | Conj 2 | Conj 3 |
|---|---|---|---|---|---|
| | | | N moles/min | | |
| $^{3}$H-GSH-5 mM | 0.5 mM | + | 2.4 ± 0.1 | 4.3±0.5 | 2.7 ±0.5 |
| No GSH | 0.5 mM | + | 0 | 0 | 0 |
| $^{3}$H-GSH-5 mM | 0 | + | 0 | 0 | 0 |
| $^{3}$H-GSH-5 mM | 0.5 mM | 0 | 2.5 | 2.6 | 1.6 |
| $^{3}$H-GSH-5 mM | 0.05 mM | + | 1.3 ± 0.2 | 1.5 ± 0.4 | 1.1 ± 0.3 |
| No GSH | 0.05 mM | + | 0 | 0 | 0 |
| $^{3}$H-GSH-5 mM | 0 | + | 0 | 0 | 0 |
| $^{3}$H-GSH-5 mM | 0.05 mM | 0 | 1.2 | 1.1 | 0.9 |

[a] Incubations were prepared on ice in a total volume of 1 mL and contained 5 mM glutathione-(glycine-2-$^{3}$H) (New England Nuclear, Boston, MA) at 82 dpm/nmole, 5 CDNB units glutathione transferase (affinity column purified from mouse liver), and 0.1 M sodium phosphate buffer, pH 8.0. Incubation vessels were transferred from the ice bath to a 37° C shaking incubator, and naphthalene oxide (either 0.5 mM or 0.05 mM) was added in 5 µL ethanol 2 min later. After 30 min, the incubation flasks were placed on ice and 2 mL ice cold methanol was added to stop the reaction. Values are the mean ± S.E. for 3 incubations or were derived from a single determination.

Resubmission of Purified Napthalene Glutathione Adducts for NMR and FAB/MS Identification

Additional quantities of these metabolites have been purified in a further attempt at identification. Both Dr. Castagnoli and Dr. Sidney Nelson (Professor of Medicinal Chemistry, University of Washington , Seattle) will be involved in the interpretation of the spectra. Sufficient quantities of the material will be purified for $^{13}$C-NMR. Adducts were prepared by stirring 0.25 mmoles (±) -naphthalene-1,2-oxide with glutathione (0.5 mmoles) in 0.1 M NaOH/Ethanol (10:1). The reaction mixture was allowed to stir for 2 hours and then was acidified to neutrality by the dropwise addition of 0.1 M HCl. The reaction mixture was then evaporated under vacuum to leave an oil. This oil was dissolved in water, and 10 µL was chromatographed repetitively on $C_{18}$-5µ Novapak columns (8 x 100 mm) with 5% acetonitrile/1% acetic acid/94% water as the mobile phase. Conjugates 1, 2, and 3 were collected, neutralized by the addition of concentrated ammonium hydroxide, and evaporated under vacuum. These were rechromatographed to yield compounds that were homogeneous by HPLC. The GSH adducts were neutralized by the dropwise addition of ammonium hydroxide and then were evaporated. Desalting was done by chromatography on a $C_{18}$ column eluted with water at 2 mL/min. Each of the conjugates eluted at 17.5 min. These were collected, evaporated in precleaned vials, and concentrated to 2 mL. A 10 µL aliquot of each of the adducts was injected onto the $C_{18}$ column and the purity of adducts 1 and 2 (based on the elution of UV absorbing substances) was estimated to be greater than 99.8%. Because there were only small amounts of adduct 3 formed in the reaction mixture, the quantities available after these purification steps were judged to be

insufficient for proper analysis. Additional quantities of adduct 3 are currently being prepared for analysis so that at least FABMS can be obtained.

The negative ion fast atom bombardment mass spectra of glutathione adducts 1 and 2 are shown in Figures 3 and 4 and tentative assignments of the major fragments are given in Table 2. The fragmentation patterns of both spectra are consistent with the presence of monoglutathione adducts of naphthalene. Prominent peaks are present in both spectra at m/z 450 which would correspond with the intact glutathionyl-naphthalene moiety. In glutathione adduct 1, there is a prominent peak at m/z 472 due to the intact naphthalene-GSH + Na. In both spectra, fragments are present at m/z 432 which would most likely correspond to a dehydration of the hydroxydihydroglutathionyl adduct to a fully aromatized thiol adduct. Both compounds yielded fragments at m/z 306 which corresponds to the intact glutathionyl moiety. This fragment has been reported by Moss et al. (1983) in the spectra of a glutathione adduct of Aflatoxin $B_1$ and by Nelson et al. (1981) in the spectra of the glutathione adduct of acetaminophen. The fragment noted at m/z 191 in adduct 1 probably represents an S-methyl naphthalene while the fragment at 159 (base peak for adduct 1) probably corresponds to S-naphthalene. Prominent peaks were observed in both spectra at m/z 128/129 and these correspond to the loss of the glutamate (minus the hydroxyl) from glutathione. Again, similar ions were noted by Moss et al. (1983) and Nelson et al. (1981). The base peak of glutathione adduct 2 (127) probably represents naphthalene.

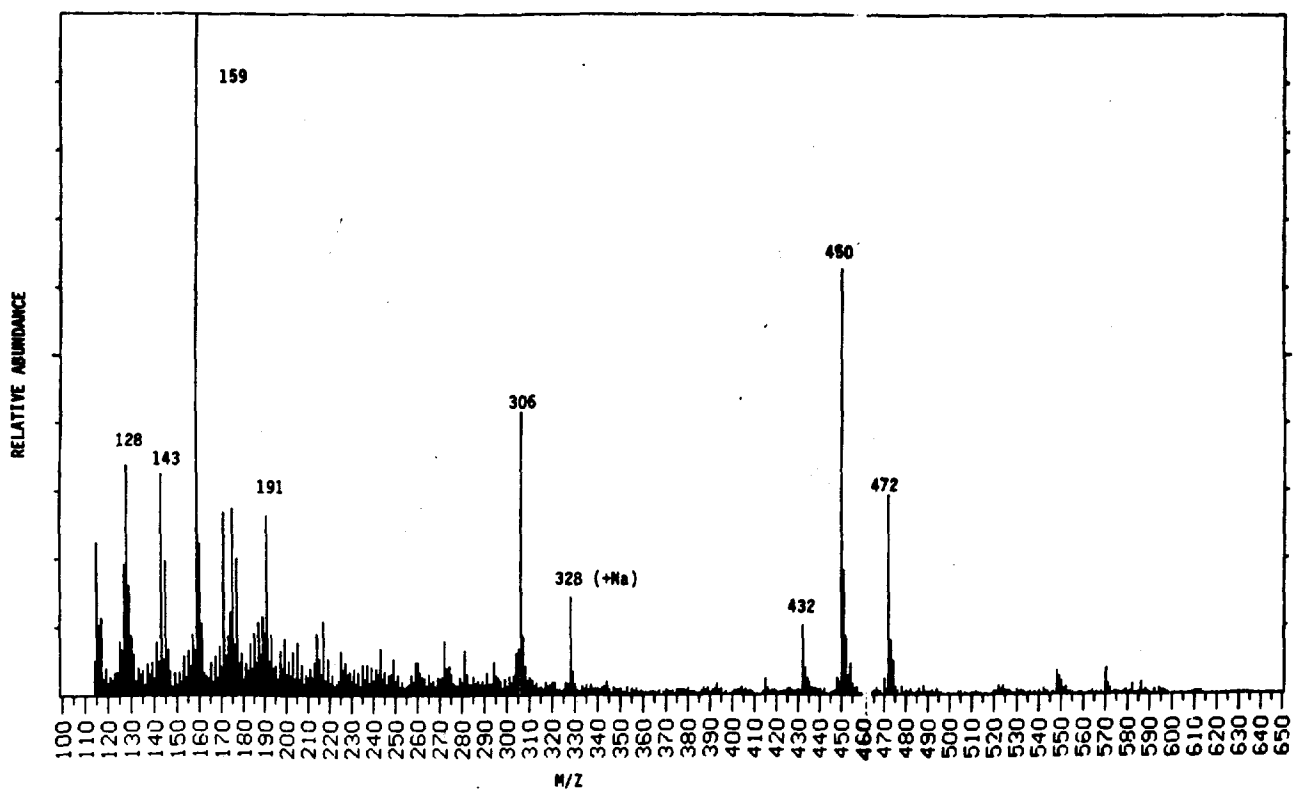



Figure 3. Fast atom bombardment mass spectra of naphthalene-glutathione adduct 1.

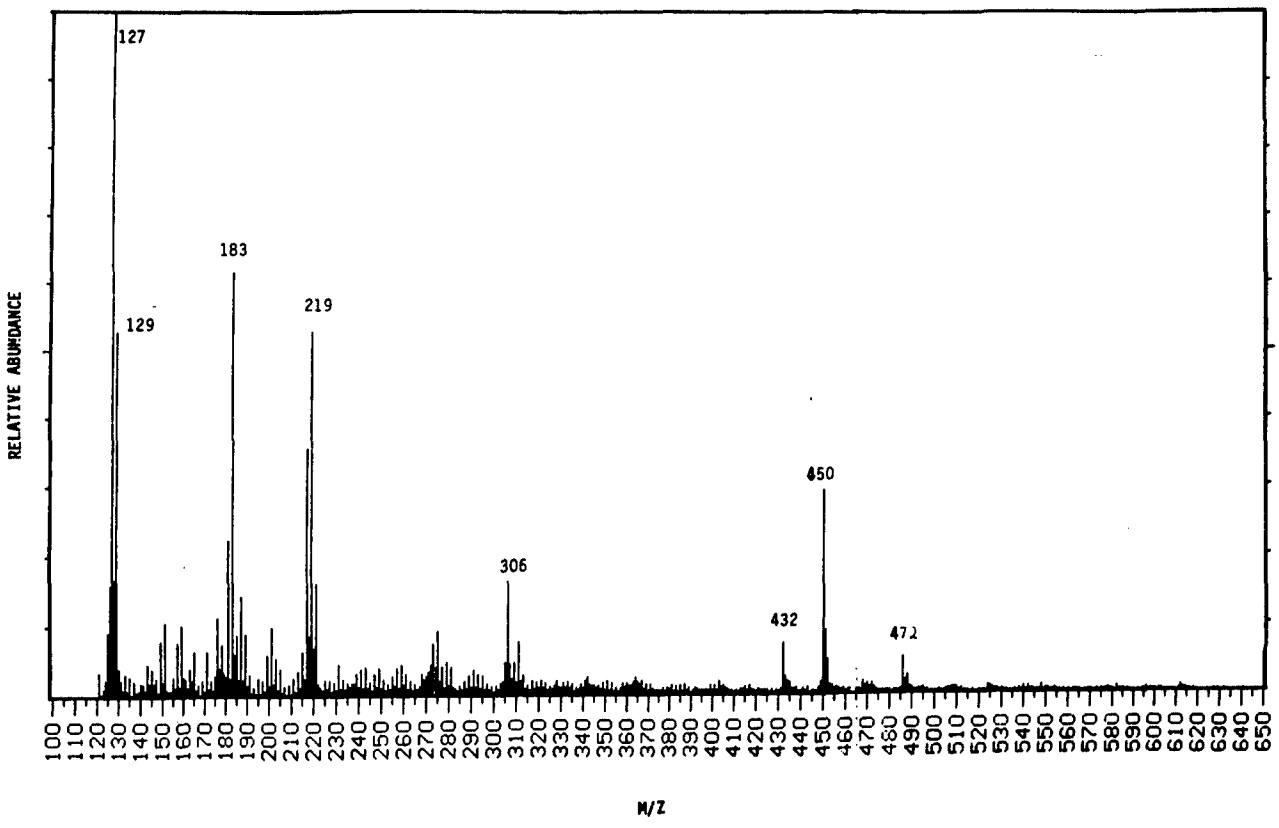
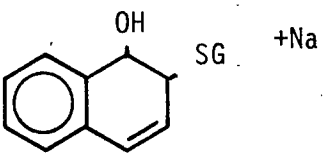
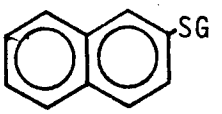
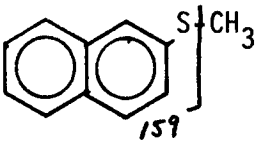
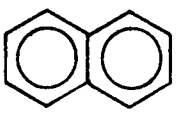



Figure 4. Fast atom bombardment mass spectra of naphthalene glutathione adduct 2.

Table 2

Major Ions in the Negative Surface Ionization Mass Spectra of Naphthalene Glutathione Adducts 1 and 2

| ADDUCT # | M/Z | RELATIVE ABUNDANCE | ASSIGNMENT |
|---|---|---|---|
| 1 | 472 | 29 |   |
| | 450 | 63 | |
| | 432 | 11 |   |
| | 328 | 13 | **GSH + Na** |
| | 306 | 42 | **GSH** |
| | 191 | 25 |   |
| | 159 | 100 | |
| | 128/129 | 33 | **GLUTAMATE (-OH)** |
| 2 | 450 | 25 | |
| | 432 | 8 | |
| | 306 | 15 | **GSH** |
| | 128/129 | 52 | **GLUTAMATE (-OH)** |
| | 127 | 100 |  |

**Comparative Studies on the Kinetics of Formation of Naphthalene-Glutathione Adducts in Microsomes of Target and Nontarget Tissues**

A major effort has been made in the 1983-84 contract year to complete the studies on the kinetics of formation of the three GSH adducts formed microsomally in target and nontarget tissues of the mouse and in lung microsomes from sensitive and nonsensitive species. There is substantial evidence, illustrated by the studies with the pulmonary toxicant, 4-ipomeanol, to suggest that the kinetics of reactive intermediate formation may play a critical role in determining the target organ for damage. For example, at saturating substrate concentrations, metabolism of 4-ipomeanol to covalently bound products in rat, mouse and guinea pig lung and liver microsomal incubations occurred at similar rates (Dutcher and Boyd, 1979) and thus did not reflect the relative sensitivity of lung vs. liver for 4-ipomeanol-induced cytotoxicity. Yet complete kinetic analyses indicated that the $K_m$ for the covalent binding reaction in rat lung microsomes was 10 fold lower than in liver microsomes (Boyd et al., 1978). Similarly, the $K_m$ for covalent binding in rabbit lung microsomes was approximately half the $K_m$ in liver microsomes (Wolf et al., 1982). These kinetic data are consistent with the relative ratios of covalent binding in lung and liver of the rat and rabbit in vivo.

Linearity in the Formation of Naphthalene Glutathione Adducts in Rat and Hamster Lung Microsomal Incubations with Time and Microsomal Protein

To establish appropriate conditions for comparing the kinetics of naphthalene-glutathione adduct formation in rat, mouse and hamster lung microsomal incubations, linearity with time and with microsomal protein was determined in incubations of rat and hamster lung microsomes.

The data shown in Tables 3 and 4 indicate that rat lung microsomes metabolize naphthalene to intermediate(s) which form glutathione adducts at easily detectable rates. The ratio of rates of formation of adduct 2/1 and adduct 2/3 were 1.7 to 1 and 1.5 to 1, respectively. The formation of all three glutathione adducts proceeded at a rate which was linear for the entire 12 minute period studied. The data in Table 4 indicate that, within experimental error, the formation of naphthalene dihydrodiol and all three glutathione adducts was linear with the quantity of microsomal protein in the incubation over the entire range of concentrations studied. Thus, incubations for the kinetic studies were 6 min. duration and contained 1 mg microsomal protein.

Table 3

Time Course Formation of Naphthalene Glutathione Adducts in Rat Lung Microsomal Incubations

| Incubation Time | Nmoles/Mg Protein[a] Conjugate 1 | Conjugate 2 | Conjugate 3 |
|---|---|---|---|
| 1 | 0.81 (n=2) | 1.99 | 0.98 |
| 2 | 1.29±0.08 | 2.41±0.10 | 1.55±0.03 |
| 4 | 1.81±0.32 | 3.63±0.90 | 1.82±0.61 |
| 8 | 3.83±0.18 | 7.11±0.45 | 4.18±0.21 |
| 12 | 5.69±0.28 | 9.95±0.54 | 6.32±0.45 |
| Regression Coef | 0.997 | 0.998 | 0.992 |

[a] Microsomal incubations were prepared on ice and contained: microsomes (1 mg), NADPH-generating system, reduced glutathione (5mM), and 10 CDNB units mouse liver glutathione transferase (partially purified by affinity chromatography). The vessels were preincubated for 2 min at 37°C, $^{14}$C-naphthalene (1.0 mM 1152 dpm/nmole) was added in 10 µL methanol and the incubation vessels were capped and incubated for the specified time. The reaction was terminated by transferring the vessels to the ice bath followed by the addition of 4 mL ice-cold methanol. Conjugates were quantified by HPLC as described previously. Values are the mean ± S.E. for 3 incubations and are reported on the basis of activity per mg microsomal protein.

Table 4

Linearity of Formation of Polar Naphthalene Metabolites with Rat Lung Microsomal Protein[a]

| Mg Micro Prot | Nmoles/Min Dihydrodiol | Conj 1 | Conj 2 | Conj 3 |
|---|---|---|---|---|
| 0.5 | 0.01 | 0.30 | 0.59 | 0.36 |
| 1.0 | 0.08 | 0.57 | 1.12 | 0.72 |
| 2.0 | 0.24 (.999)[b] | 1.04 (.998) | 1.94 (.996) | 1.33 (.965) |
| 3.0 | 0.54 (.984) | 1.47 (.998) | 2.61 (.994) | 1.81 (.984) |
| 4.0 | 0.89 (.984) | 1.81 (.996) | 3.11 (.990) | 2.16 (.987 |

[a] Lung microsomes were prepared from male Sprague Dawley rats (Charles River). Duplicate incubations were prepared on ice and consisted of the following in a total volume of 2 mL: rat lung microsomal protein in the indicated amount, $^{14}$C-naphthalene (1 mM, 798 dpm/nmole), reduced glutathione (5 mM), 10 CDNB units of mouse liver glutathione transferase and a NADPH-generating system. Incubations were done for 6 min at 37°C.

[b] Numbers in parentheses refer to linear regression coefficients calculated from the rate vs. microsomal protein curves.

Pulmonary microsomes were prepared from Syrian Golden Hamsters by standard procedures. The data in Table 5 indicate that the formation of naphthalene glutathione adducts and naphthalene dihydrodiol is linear with time in hamster lung microsomal incubations for at least 13 min. The ratio of conjugate 2/1 and 2/3 formation was 1.4 to 1 and 1.2 to 1, respectively, thus indicating that adduct 2 is not formed preferentially in hamster lung microsomal incubations as it is in mouse lung microsomal incubations.

To determine the region of linearity with microsomal protein, incubations that contained $^{14}$C-naphthalene (1.0 mM, 2307 dpm/nmole), 6 CDNB units of glutathione transferase, 5 mM reduced glutathione, NADPH-generating system and the specified quantity of microsomal protein were done for 6 min at 37°C. The data in Table 6 indicate that the conversion of naphthalene to dihydrodiol and glutathione adducts is linear with microsomal protein concentration to at least 2 mg/incubation. The rate of formation of the dihydrodiol and conjugates 1 and 3 appears to be linear to 4 mg microsomal protein while the rate of metabolism of naphthalene to adduct 2 appears to level off above 2 mg.

Table 5

Time Course Formation of Naphthalene Dihydrodiol and Naphthalene Glutathione Adducts in Hamster Lung Microsomes.[a]

| | Nmoles/Mg Microsomal Protein | | | |
|---|---|---|---|---|
| Incubation Time | Dihydrodiol | Conj 1 | Conj 2 | Conj 3 |
| 1.5 | 0.23 | 0.39 | 0.56 | 0.32 |
| 2.0 | 0.70 | 0.81 | 1.53 | 0.84 |
| 4.0 | 0.82 | 1.55 | 3.20 | 0.92 |
| 8.0 | 2.10 | 3.04 | 4.68 | 3.22 |
| 13.0 | 4.55 | 5.97 | 8.48 | 6.34 |
| Regression Coefficient | 0.983 | 0.994 | 0.989 | 0.987 |

[a] Incubations were prepared in duplicate and consisted of: 1 mg hamster lung microsomal protein, $^{14}$C-naphthalene (1.0 mM, 2307 dpm/nmole), 8.5 CDNB units mouse liver glutathione transferase (6.7 units/mg), NADPH-generating system and glutathione (5.0 mM).

Table 6

Linearity of Naphthalene Dihydrodiol and Naphthalene-Glutathione Adduct Formation with Hamster Lung Microsomal Protein Concentration.[a]

| MG Microsomal Protein | NMoles/Min Dihydrodiol | Conj 1 | Conj 2 | Conj 3 |
|---|---|---|---|---|
| 0.5 | 0.24 | 0.36 | 0.55 | 0.38 |
| 1.0 | 0.70 | 0.60 | 0.86 | 0.74 |
| 2.0 | 2.54 | 1.27 | 1.25 | 1.53 |
| 3.0 | 4.13 | 1.77 | 1.27 | 1.95 |
| 4.0 | 5.88 | 2.06 | 1.25 | 2.34 |
| Reg. Coefficient A[b] | 0.990 | 0.998 | 0.994 | 0.999 |
| Reg. Coefficient B | 0.998 | 0.992 | 0.834 | 0.987 |

[a] Duplicate incubations were prepared in a total volume of 2 mL and consisted of $^{14}$C-naphthalene (1.0 mM, 2307 dpm/nmole), NADPH-generating system, 6 CDNB units of mouse liver glutathione transferase (6.7 units/mg), 5 mM reduced glutathione, and the specified amount of microsomal protein. Incubations were done for 6 minutes at 37°C.

[b] Linear regression coefficients calculated for the 0.5, 1, and 2 mg data (A) or the entire data set (B).

Kinetics of Naphthalene-Glutathione Adduct Formation in Target vs Nontarget Tissues of the Mouse.

To determine whether the $K_m$ or $V_{max}$ for the formation of naphthalene glutathione adducts differed substantially in lung and liver, microsomes were prepared from pulmonary and hepatic tissues of male Swiss Webster mice. Microsomal incubations were carried out for 6 min using 1 mg microsomal protein, and polar naphthalene metabolites were measured by HPLC. Michaelis constants calculated by the method of Wilkinson (1961) from the data in (Figure 5) indicate that the $K_m$ for adduct formation in both lung and liver is quite low. The rates of formation of all three glutathione adducts were at or near maximal at the three highest concentrations of naphthalene used in the study. The substrate concentrations used in this study were at or above the calculated values for $K_m$ and thus caution must be exercised in interpreting these data. Further studies using substrate concentrations closer to the $K_m$ are necessary to provide a more precise determination of $K_m$ for glutathione conjugate formation. The data in Figure 5 are consistent with our previous studies on the relative rates of formation of each of the glutathione conjugates at saturating substrate concentrations. The calculated $V_{max}$ for the formation of adducts 1 and 3 in liver microsomes was substantially higher than the $V_{max}$ for these metabolites by lung microsomes. In contrast, the $V_{max}$ for conjugate 2 formation in lung microsomes was approximately 2.5 fold higher than in liver microsomes.

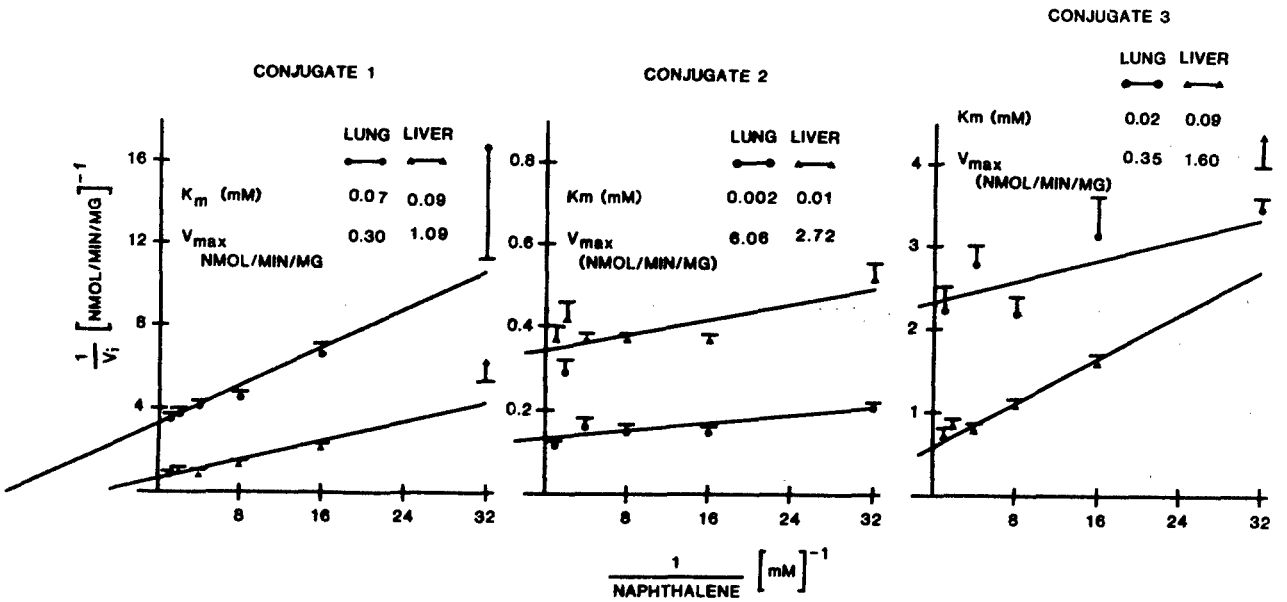



Figure 5. Double reciprocal plots comparing the formation of naphthalene glutathione adducts in microsomes from target and nontarget tissues of the mouse. Incubations were prepared in a total volume of 2.0 mL and consisted of: 1 mg microsomal protein, 10 CDNB units glutathione transferase, reduced glutathione (5 mM), NADPH generating system, and $^{14}$C-naphthalene. The following concentrations and specific activities were used: 1.0 mM - 1010 dpm/nmole; 0.5 mM - 1186 dpm/nmole; 0.25 mM - 1549 dpm/nmole; 0.125 mM - 1513 dpm/nmole; 0.0625 mM - 2418 dpm/nmole, and 0.0312 mM - 1944 dpm/nmole. Incubations were prepared on ice and were transferred to a shaking incubator for 6 min at 37°C. The incubation vessels were transferred to an ice bath, ice cold methanol was added to stop the reaction, and the dihydrodiol and glutathione conjugates were analyzed by HPLC as previously described.

The data in Table 7 indicate that the relative ratios of conjugates formed is altered with changes in substrate concentration. For example, at high substrate concentrations (>0.25 mM) the ratio of rates of formation of conjugate 2 to 1 in lung microsomal incubations was 20 to 1 and in liver microsomal incubations was 2 to 1. At low substrate concentrations, the conjugate 2 to 1 ratios were 75 to 1 in lung and 12 to 1 in liver. Likewise, the ratio of the rate of formation of conjugate 2 to 3 increased substantially in liver microsomal incubations as the substrate concentration was lowered. These apparent differences in the relative ratios of conjugates produced at high vs low substrate concentrations are consistent with $K_m$ for conjugate formation reported in Figure 5. The $K_m$ for formation of conjugate 2 is considerably lower than for conjugate 1 in the lung and is lower than for conjugate 2 and 3 in the liver. This may mean that at environmentally realistic concentrations, naphthalene is metabolized primarily to the intermediate(s) which becomes conjugate 2. These studies will require confirmation. These data suggest that different P450 isozymes may be involved in the selective formation of the intermediates that become trapped as conjugates.

Table 7

Ratios of Formation of GSH Conjugates 2 to 1 and Conjugate 2 to 3 in Hepatic and Pulmonary Microsomal Incubations Containing Varying Concentrations of Naphthalene.

| [S] mM | Conjugate 2/Conjugate 1 | | Conjugate 2/Conjugate 3 | |
|---|---|---|---|---|
| | Lung | Liver | Lung | Liver |
| 1.0 | 27.2 | 2.2 | 15.9 | 1.9 |
| 0.5 | 12.7 | 2.2 | 19.1 | 1.9 |
| 0.25 | 25.5 | 2.3 | 17.0 | 2.1 |
| 0.125 | 30.3 | 3.4 | 14.8 | 2.9 |
| 0.0625 | 43.3 | 5.4 | 20.3 | 4.2 |
| 0.0312 | 78.1 | 12 | 16.1 | 8.3 |

Kinetics of Formation of Naphthalene-Glutathione Adducts in Mouse, Rat, and Hamster Lung Microsomal Incubations.

The data in Figure 6 and Table 8 indicate that the rates of adduct 1 and 3 formation are very similar in mouse and rat lung microsomal incubations at all concentrations of naphthalene studied. In contrast, the rates of naphthalene glutathione adducts 1 and 3 formation catalyzed by hamster lung microsomes are 3 to 10 times higher than in identical rat or mouse lung microsomal incubations. Consistent with all of the previous studies on the rates of formation of each of the naphthalene glutathione adducts, the rate of formation of adduct 2 by mouse lung microsomes is 4 to 14 times higher than the rate of adduct 2 formation in rat or hamster lung microsomal incubations at saturating concentrations of substrate (Table 9). The data in Table 9 clearly indicate that not only is the total rate of adduct formation much higher (often by 4-10 fold) but that the ratio of adduct 2 to adduct 1 or 3 is considerably greater in mouse lung than in rat or hamster lung. Whether the formation of the precursor to adduct 2 is the step critical to the organ and species selective toxicity of naphthalene will require additional study but the data from the studies conducted thus far indicate that there is a good correlation between the rate of formation of adduct 2 and the organ selectivity of naphthalene-induced cytotoxicity.

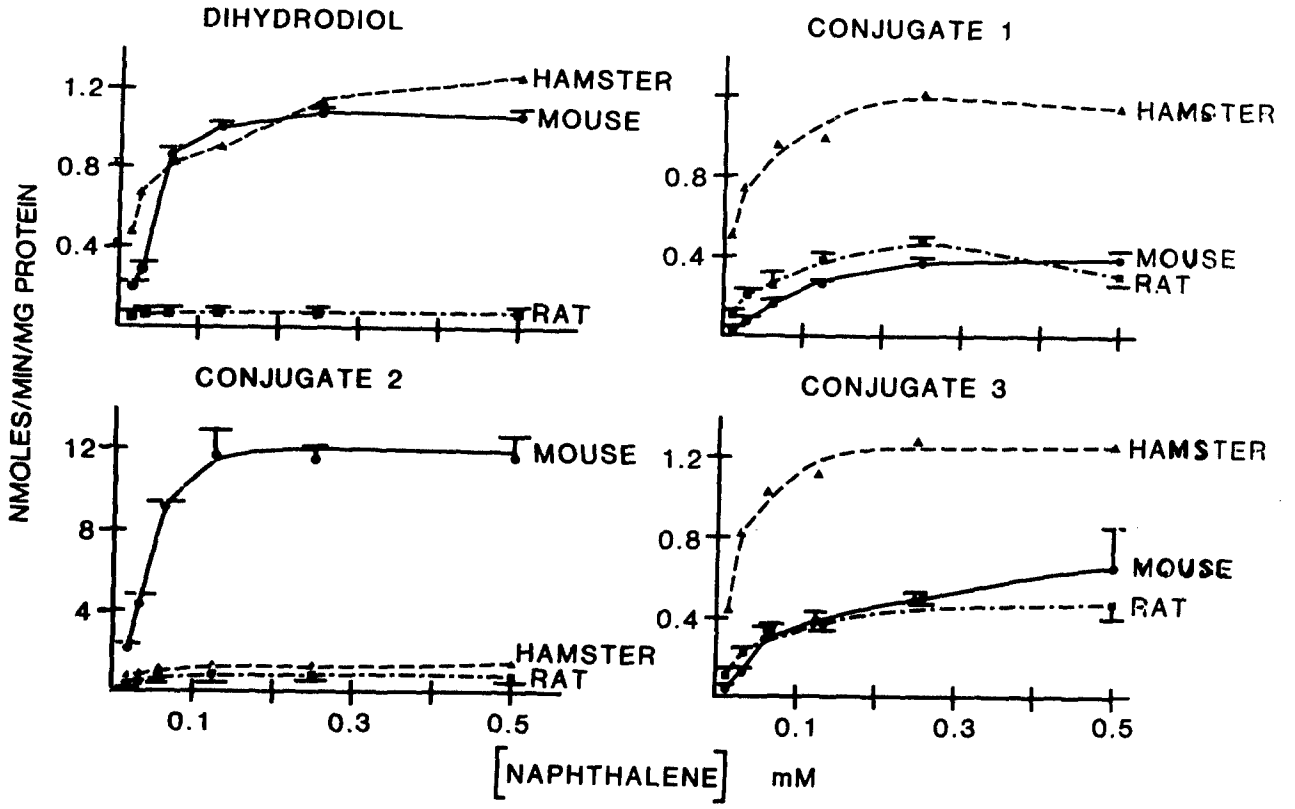



Figure 6. Comparison of the rates of metabolism of naphthalene to polar metabolites in lung microsomes from mice, rats and hamsters. Lung microsomes were prepared from male Swiss Webster mice (~30g), male Golden Syrian hamsters (~95g) and male Sprague Dawley rats (~275g) by differential ultracentrifugation. Incubations were prepared on ice and contained: 1 mg microsomal protein, 10 CDNB units of glutathione transferase prepared by affinity chromatography from mouse liver 100,000 x g supernatant (specific activity 20 CDNB units/mg), 5mM glutathione and a NADPH-generating system. Triplicate incubations with microsomes from each species were done at the following substrate concentrations and specific activities: 0.5mM - 1340 dpm/nmole; 0.25 mM - 1272 dpm/nmole; 0.125 mM - 2579 dpm/nmole; 0.0625 mM - 2535 dpm/nmole; 0.0312 mM - 5497 dpm/nmole and 0.0156 mM - 5679 dpm/nmole. Incubations were for 6 min at 37°C. Values are the mean of 2 or mean ± S.E. of three incubations. Kinetic constants were calculated by the method of Wilkinson (1961).

TABLE 8

Total Amount of Glutathione Adduct Formed and Ratios of Adducts in Mouse, Hamster and Rat Lung Microsomal Incubations at Varying Concentrations of Naphthalene

| Species | [S]mM | Total GSH Adducts Formed | Conjugate 2 / Conjugate 1 | Conjugate 2 / Conjugate 3 |
|---|---|---|---|---|
| Mouse | | | | |
| | 0.5 | 12.7 | 29.9 | 17.7 |
| | 0.25 | 12.8 | 32.2 | 24.9 |
| | 0.125 | 12.4 | 45.4 | 30.3 |
| | 0.062 | 9.6 | 55.7 | 31.3 |
| | 0.031 | 4.6 | 54.6 | 33.6 |
| | 0.015 | 2.2 | 71.0 | 53.3 |
| Rat | | | | |
| | 0.5 | 1.6 | 2.5 | 1.6 |
| | 0.25 | 1.9 | 1.8 | 1.7 |
| | 0.125 | 1.6 | 2.1 | 2.1 |
| | 0.062 | 1.4 | 3.1 | 2.3 |
| | 0.031 | 1.0 | 2.6 | 2.6 |
| | 0.015 | 0.8 | 5.3 | 4.4 |
| Hamster | | | | |
| | 0.5 | 3.9 | 1.3 | 1.2 |
| | 0.25 | 3.8 | 1.1 | 1.0 |
| | 0.125 | 3.3 | 1.1 | 1.0 |
| | 0.062 | 3.1 | 1.1 | 1.0 |
| | 0.031 | 2.4 | 1.1 | 1.0 |
| | 0.015 | 1.5 | 1.3 | 1.4 |

Table 9

Comparison of the Michaelis Constants for the Formation of Naphthalene Glutathione Adducts by Mouse, Rat and Hamster Lung Microsomes[a]

| Species | Conjugate 1 | | Conjugate 2 | | Conjugate 3 | | Total[b] | |
|---|---|---|---|---|---|---|---|---|
| | $K_m$ | V max | $K_m$ | V max | $K_m$ | V max | $K_m$ | V max |
| Mouse | 0.14 | 0.53 | 0.04 | 14.08 | 0.15 | 0.83 | 0.05 | 16.78 |
| Rat | 0.03 | 0.43 | 0.01 | 0.88 | 0.05 | 0.57 | 0.02 | 1.95 |
| Hamster | --[c] | 0.80 | 0.02 | 1.45 | 0.02 | 1.37 | 0.02 | 5.30 |

[a] Provisional and final estimates of the $K_m$ and V max were calculated by the method of Wilkinson (1961) using the data shown in Figure 6. $K_m$ is reported in mM and U max as n moles/min/mg protein.
[b] The total includes the rates of formation of naphthalene dihydrodiol plus the three glutathione conjugates.
[c] Final estimate was negative; the provisional estimate of $K_m$ was 0.12 mM.

Comparative Metabolism of Naphthalene to Glutathione Adducts in Mouse, Rat, and Hamster Lung, Liver and Kidney Microsomes.

Experiments reported thus far have compared the metabolism of naphthalene to glutathione adducts in mouse lung and liver microsomal incubations and in incubations of microsomes of rat, hamster and mouse lung. To complete these comparative studies, a final experiment was done to examine the rates of formation of the three naphthalene glutathione adducts under conditions in which the rate of reaction was maximal.

The data in Figure 7 are consistent with the reported $V_{max}$ values (Table 9) and indicate that the rate of formation of naphthalene glutathione adduct 2 in mouse lung microsomal incubations was nearly 10 fold that of adduct 2 formation in microsomal incubations prepared from lungs of nonsensitive species (rat or hamster). In contrast, the rates of formation of adducts 1 and 3 are roughly equivalent in lung microsomes from all three species studied. The rate of adduct 2 formation in mouse lung microsomal incubations was double that of mouse liver and was 4-10 fold higher than in hamster or rat liver. Consistent with previous studies, the rate of biotransformation of naphthalene to the dihydrodiol and to the glutathione adducts was very low in the kidney. Hamster kidney microsomal enzymes were the most active in metabolizing naphthalene to polar metabolites but the rate of formation of these metabolites was less than half that observed in hamster liver or lung microsomal incubations. These data provide further support for the view that target organ microsomes metabolize naphthalene to a particular intermediate (that subsequently conjugates with glutathione to form adduct 2) at substantially higher rates than microsomes of nontarget tissue and therefore that this intermediate may be an underlying basis for the organoselective toxicity of naphthalene.

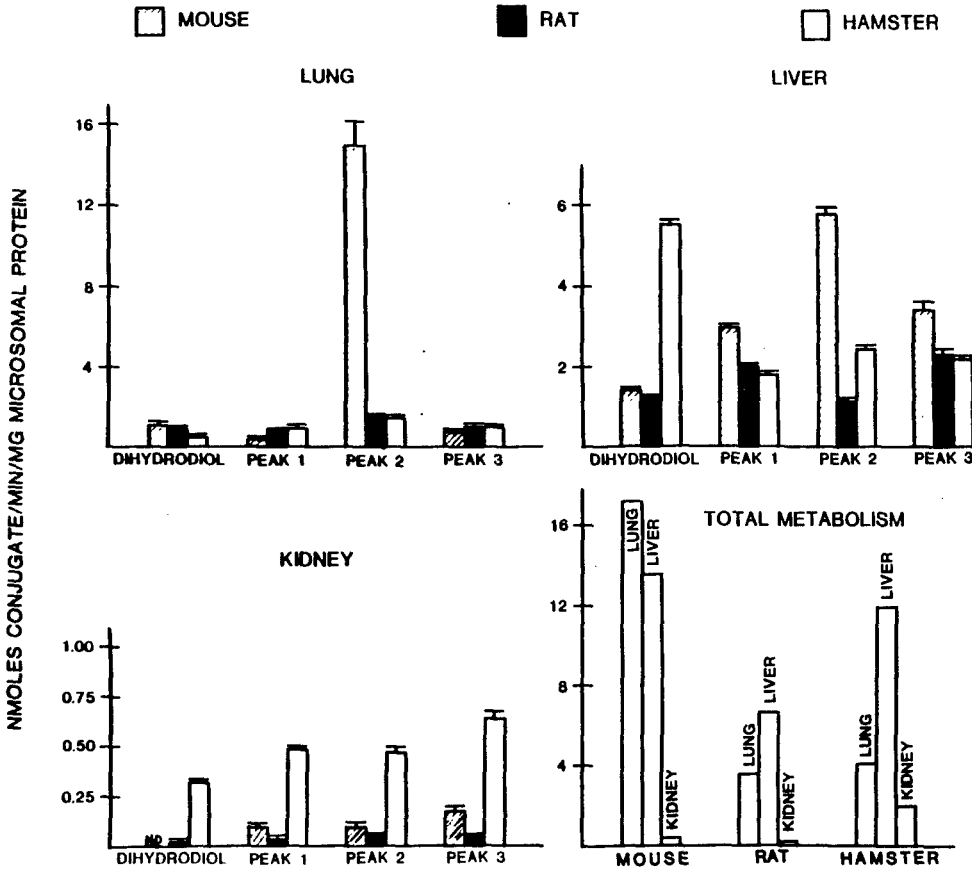



Figure 7. Comparative metabolism of naphthalene to the dihydrodiol and to three glutathione adducts in microsomal incubations of mouse, rat and hamster lung, liver and kidney. Incubations were prepared on ice and contained: 1 mg microsomal protein, $^{14}$C-naphthalene (1.0 mM, 1127 dpm/nmole), glutathione (5 mM), NADPH-generating system and 10 CDNB units of glutathione transferase activity. Incubations were conducted for 6 min at 37°C. Values are the mean ± S.E.M. for 3 incubations.

Effect of Varying Concentrations of Glutathione on Covalent Binding and Glutathione Adduct Formation in Mouse Lung and Liver Microsomes.

Studies to be discussed in more detail later in this report were designed to determine whether 1-naphthol is an obligate intermediate in the covalent binding of reactive metabolites from naphthalene. Previous work by Gillette et al. (1982) and Nelson et al. (1981) has shown that examination of the rates of covalent binding and glutathione conjugate formation at varying GSH concentrations can yield information about whether chemically reactive intermediates which bind covalently are the same as those which form glutathione adducts. (In the case of naphthalene, analysis was likely to be complicated by the fact that naphthalene-1,2-oxide, the putative precursor to the conjugates and covalently bound residues, can be metabolized via two other pathways which are markedly affected by glutathione concentration.) Further objectives of this study were to determine whether the adduct ratio (2/1 and 2/3) is altered in incubations containing different amounts of reduced glutathione and to examine the kinetics of reaction of naphthalene intermediate(s) with glutathione.

The data in Table 10 indicate that, as expected, the rate of conjugate formation increased as the concentration of glutathione in the incubations increased. Moreover, both the rate of production of the dihydrodiol and covalently bound metabolites fell as the glutathione concentration increased. In both lung and liver microsomal incubations, the rate of conjugate formation at 5.0 mM glutathione was significantly lower than at 2.5 mM glutathione. Substantial changes were not observed in the ratio of conjugates produced in either lung or liver microsomal incubations at varying glutathione concentrations (Table 11). This is consistent with earlier studies showing that the specificity of glutathione transferases plays little role in determining the ratio of conjugates produced (Buckpitt et al., 1984). These data also add support to the view that the basis for the unique pattern of glutathione adduct formation in lung microsomal incubations is due to stereoselective metabolism by pulmonary monooxygenase enzymes.

Double reciprocal plots (1/Vi vs 1/GSH) of the data presented in Table 10 indicate that the $K_m$ for adduct 1, 2 and 3 formation was 0.6 mM in the lung, and in the liver varied from 0.8 to 1.52 mM (Table 12). The difference between the $K_m$ in lung and liver is probably not significant and may be due to the variability in the data. The value for both the $K_m$ and $V_{max}$ are consistent with data obtained in previous studies. The ratio of $V_{max}$ for the formation of conjugate 2/1 and 2/3 in lungs is 23/1 and 18/1, respectively, and again is consistent with other data demonstrating the preferential formation of adduct 2 in lung microsomal incubations. Correspondingly, the ratio of $V_{max}$ for adduct 2/1 and 2/3 in liver was 2.1/1 and 1.7/1, respectively.

TABLE 10

Effect of Varying Glutathione Concentrations on the Hepatic and Pulmonary Microsomal Metabolism of Naphthalene to Covalently Bound Metabolites, to a Dihydrodriol and to Glutathione Adducts

| | | Nmoles/Min/Mg[a] | | | | |
|---|---|---|---|---|---|---|
| Tissue | GSH mM | Covalent Binding | Dihydrodiol | Conj 1 | Conj 2 | Conj 3 |
| Liver | 5.0 | 0.030±0.014 | 0.36±0.01 | 2.00±0.06 | 4.55±0.06 | 2.43±0.06 |
| | 2.5 | 0.015±0.001 | 0.88±0.06 | 2.58±0.18 | 5.58±0.22 | 2.93±0.13 |
| | 1.0 | 0.047±0.002 | 2.05±0.07 | 2.09±0.07 | 4.42±0.17 | 2.09±0.08 |
| | 0.5 | 0.121±0.011 | 3.48±0.13 | 0.96±0.01 | 2.30±0.03 | 0.72±0.01 |
| | 0.25 | 0.178±0.019 | 4.17±0.28 | 0.65±0.03 | 0.55±0.05 | 0.56±0.01 |
| | 0.125 | 0.198(n=2) | -- | -- | -- | -- |
| Lung | 5.0 | 0.004±0.002 | 0.19±0.01 | 0.27±0.01 | 6.22±0.19 | 0.38±0.02 |
| | 2.5 | 0.004±0.001 | 0.35±0.03 | 0.36±0.02 | 7.59±0.56 | 0.45±0.01 |
| | 1.0 | 0.009±0.001 | 0.81±0.04 | 0.30±0.01 | 7.29±0.33 | 0.38±0.01 |
| | 0.5 | 0.028±0.002 | 1.65±0.13 | 0.16±0.01 | 3.97±0.08 | 0.19±0.01 |
| | 0.25 | 0.101±0.002 | 2.11±0.05 | 0.11±0.01 | 2.61±0.02 | 0.15±0.01 |
| | 0.125 | 0.163±0.001 | 2.11±0.04 | 0.11 (n=2) | 2.34±0.08 | 0.14±0.01 |

[a] Pulmonary and hepatic microsomes were prepared from tissues of male Swiss Webster mice by standard procedures. Incubations, conducted for 6 min at 37°C, contained: 1 mg microsomal protein, $^{14}$C-naphthalene (1.0mM, 1292 dpm/nmole), NADPH-generating system, 10 CDNB units mouse liver glutathione transferase and reduced glutathione (0.125, 0.25, 0.50, 1.0, 2.5, or 5.0 mM). The formation of glutathione conjugates was assessed by HPLC analysis of the methanol/water supernatant, and covalent binding was measured in the precipitated protein as described previously. Values are the mean ±S.E. for 3 incubations.

TABLE 11

Ratios of Glutathione Adduct 2/1 and 2/3 in Lung and Liver Microsomal Incubations Containing Varying Concentrations of Glutathione

| Tissue | (GSH)mM | CONJ 2/1 | CONJ 2/3 |
|---|---|---|---|
| Liver | 5.0 | 2.3 | 1.9 |
| | 2.5 | 2.2 | 1.9 |
| | 1.0 | 2.1 | 2.1 |
| | 0.5 | 2.4 | 3.2 |
| | 0.25 | 2.4 | 2.8 |
| Lung | 5.0 | 23.0 | 16.4 |
| | 2.5 | 21.1 | 16.9 |
| | 1.0 | 24.3 | 19.2 |
| | 0.5 | 24.8 | 20.9 |
| | 0.25 | 23.7 | 17.4 |
| | 0.125 | 21.3 | 16.7 |

TABLE 12

Kinetic Constants for the Formation of Naphthalene-Glutathione Adducts in Mouse Lung and Liver Microsomes[a]

| | Conjugate 1 | | | Conjugate 2 | | | Conjugate 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Tissue | $K_m$ | $V_{max}$ | LRC[b] | $K_m$ | $V_{max}$ | LRC | $K_m$ | $V_{max}$ | LRC |
| Lung | 0.61 | 0.38 | 0.96 | 0.60 | 8.92 | 0.97 | 0.63 | 0.50 | 0.97 |
| Liver | 0.97 | 3.13 | 0.97 | 0.82 | 6.60 | 0.98 | 1.52 | 3.78 | 0.95 |

[a] Kinetic constants were calculated from double reciprocal plots of data obtained from incubations of lung or liver microsomes (1mg), $^{14}$C-naphthalene (1.0mM, 1292 dpm/nmole), NADPH-generating system and concentrations of glutathione ranging from 0.125 to 2.5 mM. Values reported for $K_m$ are mM and those for $V_{max}$ are nmoles/min/mg protein.

[b] LRC - linear regression coefficient.

If naphthalene-1,2-oxide is an intermediate in the covalent binding of reactive metabolites from naphthalene and increasing concentrations of glutathione decrease the steady state concentration of naphthalene oxide, then plots of the amount of dihydrodiol plus naphthol vs covalent binding would be expected to be linear and should pass through the origin. If, on the other hand, naphthol is the prime intermediate in the covalent binding, plots of 1-naphthol vs covalent binding should be linear and should pass through the origin.

The data in Table 3 indicate that as glutathione concentrations in the incubation decreased, the covalent binding and formation of naphthol and naphthalene dihydrodiol increased. At the highest four glutathione concentrations studied, plots of naphthol + dihydrodiol vs covalent binding were linear (linear regression coefficients were 0.997 and 0.984 for lung and liver microsome incubations, respectively) and intersected the y axis near the origin (0.67 and 0.16, respectively). However, there was an abrupt deviation from linearity occurring at the lowest two concentrations of glutathione studied which may be due to a lack of linearity with time in the formation of the various metabolites at low glutathione concentrations when the microsomal membranes are relatively unprotected from reactive metabolites of naphthalene. Thus, while these data do not preferentially support our hypothesis that naphthalene-1,2-oxide is an intermediate in the covalent binding of radioactivity from $^{14}C$-naphthalene in vitro, they are consistent with the possibility that naphthalene-1,2-oxide may contribute to the overall levels of bound metabolites.

Table 13

Formation of 1-Naphthol, Naphthalene Dihydrodiol, and Covalently Bound Metabolites in Mouse Lung and Liver Microsomal Incubations Containing Varying Concentrations of Glutathione

| Tissue | [GSH] mM | 1-Naphthol | Dihydrodiol | Covalent Binding |
|---|---|---|---|---|
| | | | NMoles/Min/Mg | |
| Lung | 5.0 | 1.21 | 0.19 | 0.031 |
| | 2.5 | 1.44 | 0.36 | 0.021 |
| | 1.0 | 2.08 | 0.80 | 0.061 |
| | 0.5 | 5.59 | 1.65 | 0.17 |
| | 0.25 | 6.40 | 2.11 | 0.61 |
| | 0.125 | 5.68 | 2.11 | 0.98 |
| Liver | 5.0 | 1.29 | 0.36 | 0.18 |
| | 2.5 | 1.18 | 0.88 | 0.09 |
| | 1.0 | 2.19 | 2.05 | 0.28 |
| | 0.5 | 6.62 | 3.47 | 0.72 |
| | 0.25 | 5.59 | 4.17 | 1.07 |
| | 0.125 | 8.53 | - | - |

Incubation of Human Lung Microsomes with Naphthalene, Glutathione, and Glutathione Transferases.

The objectives of the studies in human lung tissue were to fully characterize the metabolism of naphthalene to reactive metabolites, to determine whether the nature and rate of formation of these metabolites is similar to that observed in target or nontarget rodent tissues, and to determine whether there is an "inhibitor" present in human lung microsomes which could potentially control the rate at which xenobiotics are oxygenated.

Three fresh specimens of human pulmonary tissue have been obtained through the Pathology Department, St. Joseph's Hospital, Orange, California. The tissue was placed in ice cold Hepes buffered balanced salt solution within 60 minutes of removal from the patient. The preparation of pulmonary microsomes was started within two hours of excision of the tissue and incubations were prepared using the method currently used for rodent lung microsomes.

The data in Figure 8 were derived from the 60 year old female and they indicate that the metabolism of naphthalene to the dihydrodiol occurs at a rate which is nearly double the rate of formation of any of the glutathione adducts. The rates of formation of the dihydrodiol and conjugate 1 appear to be linear for 20 min while the rates of formation of adducts 2 and 3 appear to level off after 10 min. Thus, the linearity of metabolism of naphthalene to dihydrodiol and glutathione adducts appears similar to that observed in microsomes from rodents, and incubation times will have to be no longer than 10 min to be appropriate for the measurement of initial velocities.

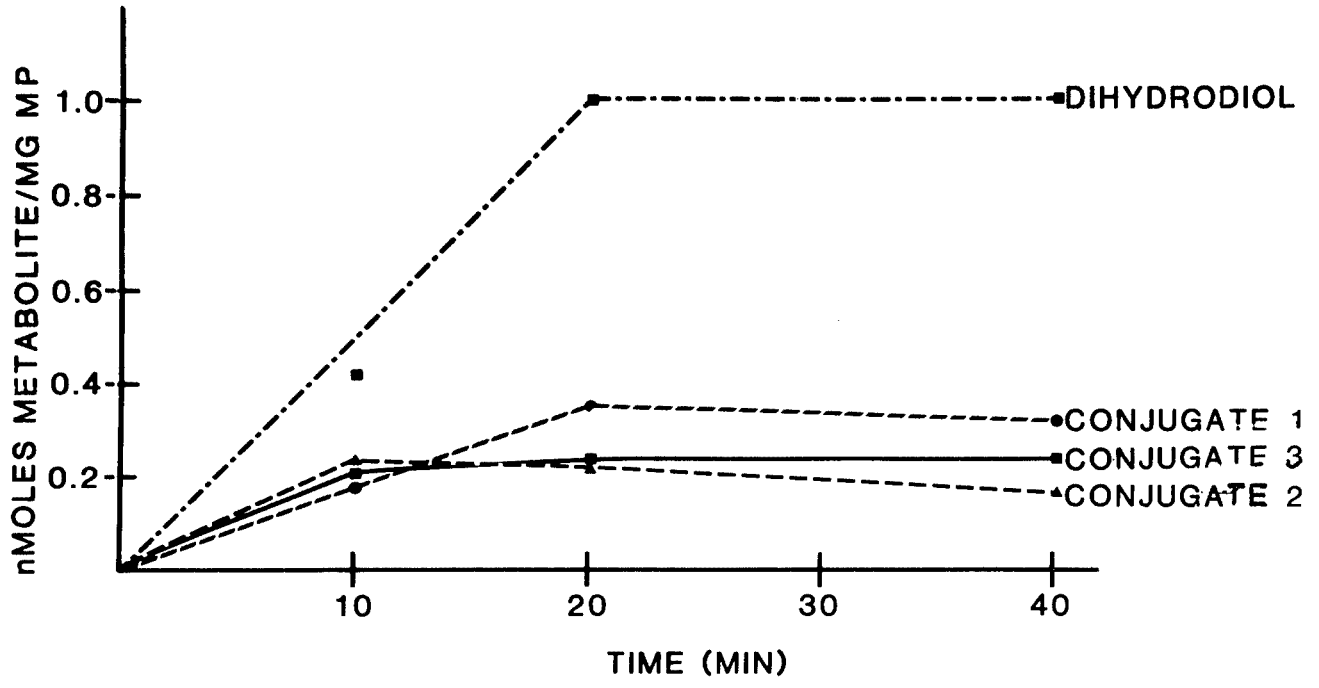



Figure 8. Time course metabolism of naphthalene by human lung microsomes. Incubations contained in a total volume of 2 mL: $^{14}$C-naphthalene (1mM, 1557 dpm/nmole) glutathione (5 mM), NADPH-generating system, 10 CDNB units glutathione transferase activity and 3.65 mg human lung microsomal protein. Each point represents the mean of 2 incubations.

There are several interesting points about the data shown in Figure 8. First, the fact that the rate of dihydrodiol formation is considerably higher than glutathione conjugate formation is in marked contrast to what is observed in lung microsomes from the rat, mouse, and hamster and is consistent with the high activities of epoxide hydrolase that have been reported in human lung tissue microsomes (Fakjian et al., unpublished results). In contrast to all the studies of xenobiotic metabolism in human lung microsomes where cytochrome P450 has been at or below the limit of detection (Prough et al., 1979; McManus et al., 1980; Oesch et al., 1980; Lorenz et al., 1979; Jakobsen et al., 1982) and where the rates of benzopyrene hydroxylation (Prough et al., 1979, McManus et al., 1979; Oesch et al., 1979) of ethoxycoumarin deethylation (McManus et al., 1979;

Oesch et al., 1980), aminopyrine hydroxylation (Oesch et al., 1980), and biphenyl hydroxylation (Oesch et al., 1980) have either not been detected or have been at the limits of detection, our studies have shown that the metabolism of naphthalene can be easily detected. This may be due to a number of factors. Most researchers freeze their lung tissue before use, but our studies have found that freezing mouse lung tissue for 24 hours at -80$^{o}$ C decreases the rate of metabolism of bromobenzene to glutathione adducts in lung slices by approximately 75% in comparison with fresh tissue (Fakjian et al., unpublished results). Moreover, freezing rat lung microsomes in suspension for 9 days at - 80 $^{o}$ resulted in a decrease in the ability of those microsomes to form polar metabolites from naphthalene by approximately 50%. Thus, the relatively higher activities obtained in our studies may be due to the fact that the tissue was obtained fresh and used immediately.

Another point that should be noted from the data in Figure 8 is that the rates of formation of each of the conjugates are nearly identical. This pattern differs considerably from the preferential formation of adduct 2 by mouse lung and is similar to that observed in hamster or rat lung microsome incubations.

Studies from Oesch's lab (1980) have shown that the addition of human lung microsomes to microsomes from rat lung markedly decreased the rate of rat lung microsome-catalyzed deethylation of 7-ethoxycoumarin, thus suggesting that human lung microsomes contain some sort of inhibitor of the P450 monooxygenases. To confirm and extend Oesch's observations, the metabolism of naphthalene was examined in mouse liver microsomal preparations to which BSA or human lung microsomes were added. As indicated by the data in Figure 9, addition of 4 mg BSA to mouse liver microsomes produced a doubling in the rate of dihydrodiol formation but caused no change in the rates of formation of the glutathione conjugates. Addition of 1.8 mg human lung microsomes and 2 mg BSA to mouse liver microsomal incubations resulted in a sharp increase in the rate of formation of the dihydrodiol which again is consistent with the high epoxide hydrolase activities of human lung microsomes. The rates of conjugate formation were substantially decreased under these conditions. Addition of 3.6 mg human lung microsomes to mouse liver microsome incubations resulted in rates of dihydrodiol formation that were similar to those observed in comparable incubations containing 4 mg BSA. Doubling the amount of human lung microsomal protein added to the incubation from 1.8 to 3.6 mg appeared to result in a further slight decrease in the rate of formation of the glutathione adducts. Overall, the addition of increasing amounts of human lung microsomal protein appeared to result in substantial decreases in the rates of naphthalene metabolism. (Table 14)

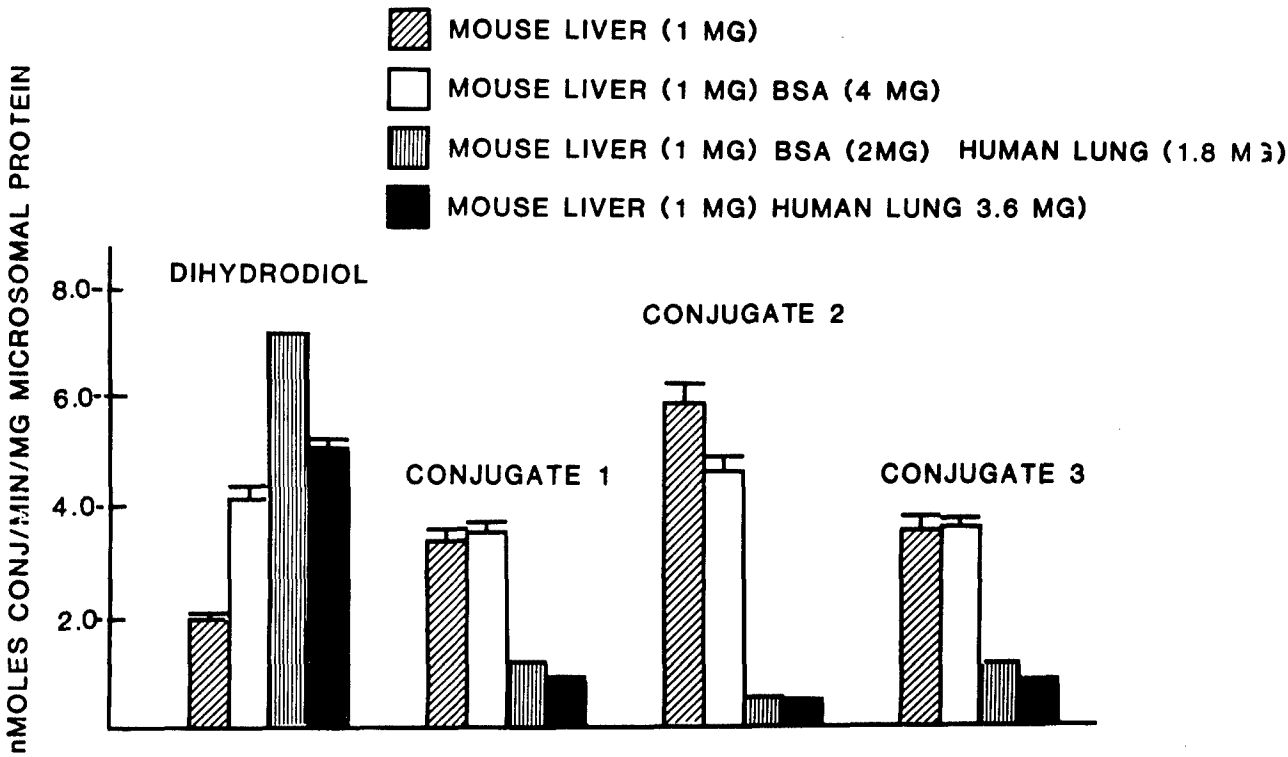



Figure 9. Effect of addition of BSA or varying amounts of human lung microsomal protein on the metabolism of naphthalene by mouse liver microsomal preparations. Mouse liver microsomes (1 mg protein) were incubated with GSH (5 mM), $^{14}$C-naphthalene (1 mM, 1557 dpm/nmole), a NADPH-generating system, 10 CDNB units of glutathione transferase, and either bovine serum albumin or human lung microsomes as indicated. Incubations were for 10 min at 37°C; values are the mean of 2 or the mean ± S.E. of three incubations and are based on the quantity of mouse liver microsomal protein in the incubation.

Table 14

Effect of Addition of Human Pulmonary Microsomes on Mouse Liver Microsome-Mediated Naphthalene Metabolism.

| Mouse Liver Microsomes | BSA | Human Lung Microsomes | Rate of Naphthalene Metabolism[a] (nmoles/min) |
|---|---|---|---|
| 1 mg | - | - | 20.3 |
| 1 mg | 4 mg | - | 21.8 |
| 1 mg | 2 mg | 1.8 mg | 13.8 |
| 1 mg | - | 3.6 mg | 9.6 |

[a] The rate of naphthalene metabolism was calculated by adding the total amount of dihydrodiol and GSH adducts produced.

The second patient from whom lung was obtained was a 61 year old male smoker (1/2 pack/day x 40 years) who was taking no medication known to affect xenobiotic metabolism. Enough tissue was available so that the lung was divided into bronchiolar, parenchymal, and tumor areas. Tumor tissue was extremely tough and could not be completely homogenized. The intent of this second experiment was to confirm the time course results obtained previously, to achieve some estimate of the interindividual variability, to attempt to shunt all of the naphthalene oxide formed into conjugates (by including the epoxide hydrolase inhibitor, cyclohexene oxide), and to confirm the inhibition of mouse liver microsome-mediated conjugate formation by addition of human lung microsomes.

The data in Table 15 indicate that metabolism of naphthalene to the glutathione adducts by parenchymal lung microsomes was linear for 20 min. There was a moderate amount of variability in duplicate incubations from many of the time points studied and this is probably attributable to the low activities observed in this experiment. In contrast to the previous piece of lung tissue obtained for studies of naphthalene metabolism, this specimen showed microsomal naphthalene metabolizing activity (i.e., total dihydrodiol plus conjugate formation) of less that 20% of the first sample. This difference may be due to several factors such as age, sex, or diet. It should also be noted that a relay on the ultracentrifuge failed during the first 100,000 x g centrifugation and by the time the rotor could be stopped the samples had reached a temperature of $8^{o}$ C. While this did not appear to have a marked effect on mouse liver microsomal activity, it is entirely possible that the monooxygenases in human lung are far more heat labile than the enzymes in mouse liver. In addition, a comparison of the rates of adducts produced in the last experiment (1:1:1) with this experiment (1:6:1) indicate the formation of adduct 2 was far higher relative to 1 or 3. Again this could be due to several factors - interindividual variability in the stereoselectivity of attack by the pulmonary monooxygenases or the fact that cyclohexene oxide was added to these incubations. Addition of this epoxide hydrolase inhibitor had previously been shown to selectively increase the rate of adduct 2 formation in mouse liver microsomal incubations. (Buckpitt et al., 1984)

Another factor that is evident from these studies is that addition of the epoxide hydrolase inhibitor, cyclohexene oxide, resulted in nearly complete inhibition of the formation of naphthalene dihydrodiol, and this will be an effective method for shunting all of the reactive metabolites to glutathione adducts. The other point which should be noted is that there are not major differences in the metabolism of naphthalene between the rather crudely divided areas of lung. Our methods of separating these areas will have to be refined before a definitive conclusion can be reached, however.

Table 15

Time Course Formation of Naphthalene Dihydrodiol and Naphthalene Glutathione Adducts in the Microsomes Prepared From Human Bronchial, Parenchymal and Tumor Lung Sections

| Metabolite | Time | Nmoles/Mg Microsomal Protein [a] Parenchyma | Bronchial | Tumor |
|---|---|---|---|---|
| Dihydrodiol | 5 | 0.007 | N.D.[b] | N.D. |
| | 10 | 0.016 | N.D. | ---- |
| | 15 | ----- | 0.008 | ---- |
| | 20 | 0.032 | ----[c] | ---- |
| GSH Adduct 1 | 5 | 0.024 | 0.030 | .022 |
| | 10 | 0.020 | 0.038 | ---- |
| | 15 | 0.049 | 0.021 | ---- |
| | 20 | 0.078 | ----- | ---- |
| GSH Adduct 2 | 5 | 0.091 | 0.093 | 0.054 |
| | 10 | 0.018 | 0.120 | ---- |
| | 15 | 0.184 | 0.128 | ---- |
| | 20 | 0.446 | ----- | ---- |
| GSH Adduct 3 | 5 | 0.025 | 0.022 | 0.022 |
| | 10 | 0.021 | 0.038 | ---- |
| | 15 | 0.046 | 0.022 | ---- |
| | 20 | 0.094 | ----- | ---- |

[a] Values are the means of 2 incubations with the exception of the 10 and 20 min parenchymal lung which were values obtained from a single incubation. Incubations contained in a total volume of 2 mL: $^{14}$C-naphthalene (1.0 mM, 1557 dpm/nmole), 4 mg human lung microsomal protein, reduced glutathione (5.0 mM), 10 CDNB units GSH transferase (8.1 CDNB units per mg), NADPH-generating system and 0.5 mM cyclohexene oxide.
[b] N.D. not detected.
[c] —— not done.

The data in Table 16 contrast markedly with that obtained in the previous experiment which showed substantial inhibition of mouse liver microsomal metabolism of naphthalene by addition of human lung microsomes. No significant decrease in the formation of any of the glutathione adducts was observed when human lung microsomes were added to mouse liver microsomes while there was a slight increase in the rate of dihydrodiol formation. Thus, the data generated in this experiment differ in two important aspects from the data obtained in a previous study with human lung. The ratio of adducts formed by lung microsomes in this study was 1:6:1; these ratios are closer to those observed in target tissue microsomes than in the first experiment with human lung where the ratio of adducts was 1:1:1. Moreover, in the second experiment, addition of human lung microsomes to mouse liver microsomal incubations did not result in a significant decrease in the rate of adduct formation.

Table 16

Effect of Human Lung Microsomes on the Rate of Naphthalene Metabolism by Mouse Liver Microsomes

| Mouse Liver Microsomes (mg) | BSA (mg) | Human Lung Microsomes (mg) | Nmoles/mg Mouse Liver m.p./min Diol | Conj 1 | Conj 2 | Conj 3 |
|---|---|---|---|---|---|---|
| 1 | 0.0 | 0.0 | 0.07 ±0.01 | 2.94 ±0.12 | 5.97 ±0.07 | 3.40 ±0.06 |
| 1 | 4.0 | 0.0 | 0.07 ±0.01 | 3.19 ±0.22 | 6.76 ±0.24 | 3.82 ±0.14 |
| 1 | 3.2 | 0.8 | 0.15 ±0.02 | 2.78 ±0.21 | 6.09 ±0.18 | 3.32 ±0.16 |
| 1 | 1.6 | 2.4 | 0.33 | 3.17 | 6.08 | 3.38 |

[a] Incubations contained in a total volume of 2 mL: $^{14}$C-naphthalene (1.0 mM, 1557 dpm/nmole), reduced glutathione (5.0 mM), NADPH-generating system, 0.5mM cyclohexene oxide (added in 10 µL acetonitrile), 10 CDNB units of glutathione transferase, and bovine serum albumin or human lung microsomes as indicated. Incubations were prepared on ice and were transferred to a shaking incubator for 10 min at 37° C.

Microsomes from a third specimen obtained from a 77-year-old male cigar smoker were prepared by standard techniques. In previous studies (Table 14, Figure 9), substantial inhibition of mouse liver microsome-catalyzed metabolism of naphthalene was noted upon the addition of human lung microsomes. In an effort to remove this potential "inhibitory substance", half of the microsomal pellet from the first 100,000 x g centrifugation was resuspended in 20 mL Tris/KCl buffer containing 10% glycerol and was applied to a 40 x 3.5 cm column packed with Sepharose 2B. (This procedure had previously been shown by Capdevila et al. (1975) to be very efficient in removing hemoglobin from microsomal preparations.) The column was eluted with Tris/KCl/10% glycerol and the fractions eluting in the column void volume were collected and centrifuged at 100,000 x g for collection of the microsomal pellet. The other half of the microsomal pellet collected after the first 100,000 x g centrifugation was washed and pelleted two more times.

The data in Table 17 indicate that microsomes from this specimen of human lung metabolized naphthalene to the dihydrodiol and all three glutathione adducts at an easily measurable rate, albeit at a rate that is considerably lower than that observed in preparations of rodent lung microsomes. In comparison with the data obtained previously with human lung, the rate of polar naphthalene metabolite formation with this sample was slightly higher than the first sample obtained and considerably higher than the second (Table 18). In contrast to the marked inhibition of mouse liver-catalyzed naphthalene metabolism observed when microsomes from the first specimen of human lung were added to the incubations, no decrease in the rate of formation of polar naphthalene metabolites was noted in this experiment (Table 17). The underlying reasons for the differences in results obtained with the first specimen of human lung which demonstrated a marked inhibition of mouse liver microsome-catalyzed metabolism of naphthalene and the last two experiments where no inhibition was observed are currently unclear. The epoxide hydrolase inhibitor, cyclohexene oxide, has been included in the past two incubations and was not in the first. It is possible that there are other enzymes in human lung microsomes capable of catalyzing the rapid metabolism of the dihydrodiol if formed and thus the further metabolism of this yielded

the rapid metabolism of the dihydrodiol if formed and thus the further metabolism of this yielded a falsely low estimation of the rate of naphthalene metabolism. On the other hand, the differences between these preparations could simply be due to interindividual differences in the components isolated with the microsomal fraction.

The other point that should be noted from the data in Table 17 is that chromatography of the microsomal fraction over the Sepharose column resulted in a microsomal fraction with no detectable activity. This contrasts sharply with the data from Capdevila et al. (1975) who showed that this method resulted in much higher specific contents of rat lung cytochrome P450.

Table 17

Metabolism of Naphthalene to Polar Metabolites by Human Lung Microsomes[a]

| Mg Micro. Prot | | BSA | Nmoles/Mg Microsomal Protein/10 min | | | | |
|---|---|---|---|---|---|---|---|
| Human | Mouse | | Diol | Conj 1 | Conj 2 | Conj 3 | Total |
| 4.0[b] | 0.0 | 0.0 | N.D. | N.D. | N.D. | N.D. | |
| 4.0 | 0.0 | 0.0 | 0.011 | 0.036 ±.002 | 0.061 ±.014 | 0.027 ±.020 | 0.135 ±.006 |
| 0.0 | 1.0 | 0.0 | 0.119 | 1.926 ±.007 | 6.690 ±.028 | 4.090 ±.092 | 12.71 ±.066 |
| 0.0 | 1.0 | 4.0 | 0.129 | 1.694 ±.012 | 5.635 ±.140 | 3.702 ±.124 | 11.03 ±.358 |
| 4.0 | 1.0 | 0.0 | 0.940 | 1.638 ±.023 | 5.885 ±.090 | 3.349 ±.084 | 10.87 ±.114 |

[a] Incubations were prepared on ice and contained in a total of two mL: microsomal protein (as indicated), glutathione (5mM), cyclohexene oxide (0.5 mM), NADPH-generating system, 10 CDNB units of semi-purified glutathione transferase activity (mouse liver), and $^{14}$C-naphthalene (1.0 mM, 1410 dpm/nmole). Incubations were conducted for 10 min at 37°C and naphthalene dihydrodiol and the glutathione conjugates were analyzed by HPLC. Data are the mean ±S.E. for three incubations. Experiment done 6/84.

[b] Sepharose microsomes.

Table 18

Interindividual Differences in the Rates of Naphthalene Metabolism by Microsomes from Human Lung

| Sample | Date | Smoker | Sex | Age | NMoles/mg Microsomal Protein | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Diol | Conj 1 | Conj 2 | Conj 3 | Total |
| 1 | 2/84 | ? | F | 60 | 0.42 | 0.17 | 0.24 | 0.21 | 1.04 |
| 2 | 3/84 | Y | M | 61 | 0.01 | 0.03 | 0.12 | 0.30 | 0.18 |
| 3 | 6/84 | Y | M | 77 | 0.11 | 0.36 | 0.61 | 0.27 | 1.35 |

All incubations were for 10 min at 37°C and contained the following in a total of 2 mL: human lung microsomal protein (3.6-4.0 mg), NADPH-generating system, $^{14}$C-naphthalene (1.0 mM), glutathione (5 mM) and partially purified glutathione transferases (10 CDNB units).

**Intermediacy of 1-Naphthol in the Covalent Binding of Reactive Metabolites from Naphthalene**

Studies with benzene, bromobenzene, and naphthalene have suggested that the cytochrome P450-mediated metabolism of these aromatic hydrocarbons to covalently bound metabolites involves the intermediate formation of a phenol (Hesse and Metzger, 1979; Tunek et al., 1980; Sawahata et al., 1983; Lunte et al., 1983). The data supporting these conclusions include the demonstration that: 1) the disappearance of the parent compound correlates with the formation of the monohydroxylated derivative and that covalent binding appears to lag behind these processes; 2) that the monohydroxylated derivative can be metabolized to covalently bound metabolites; and 3) that addition of glutathione to microsomal incubations of benzene or phenol results in the formation of the same glutathione adducts. While these data are consistent with the view that the monohydroxylated hydrocarbons are intermediates in the process of covalent binding, the data do not exclude the possibility that a portion of the bound metabolite arises from metabolism of the parent hydrocarbon. Indeed, recent studies by Lau et al. (1984) provide clearcut evidence that not all of the covalently bound metabolites from bromobenzene arise from *p*-bromophenol. A number of pieces of evidence have been presented earlier in this report which suggest that a portion of the reactive naphthalene metabolites becoming covalently bound to microsomal protein arise directly from naphthalene oxide. The finding that i.p. administration of $^{14}$C-1-naphthol results in levels of covalently bound metabolite similar to those observed after comparable doses of naphthalene and that 1-naphthol did not decrease tissue glutathione levels in vivo argue against the formation of this metabolite as an obligate intermediate in the covalent binding of reactive metabolites after administration of naphthalene. Studies will be presented in this progress report which further support this view.

Time Course Formation of Naphthol, Naphthalene Dihydrodiol, and Covalently Bound Metabolites in Mouse Lung and Liver Microsomal Incubations.

Previous studies showed that the rates of formation of naphthol in lung microsomes were substantially higher than in liver microsomes while the rates of formation of covalently bound metabolites in these incubations were nearly identical. An additional study was done to confirm our preliminary findings.

The data in Figure 10 indicate that the formation of 1-naphthol in lung microsomal incubations occurred at a rate that was 2.5 times the rate in liver microsomal incubations while the rate of covalent binding of radioactivity was virtually identical over the first 10 min of the

incubation. These studies continue to support the thesis that not all of the covalently bound radioactivity derived from $^{14}$C-naphthalene in vitro arises through 1-naphthol as an intermediate.

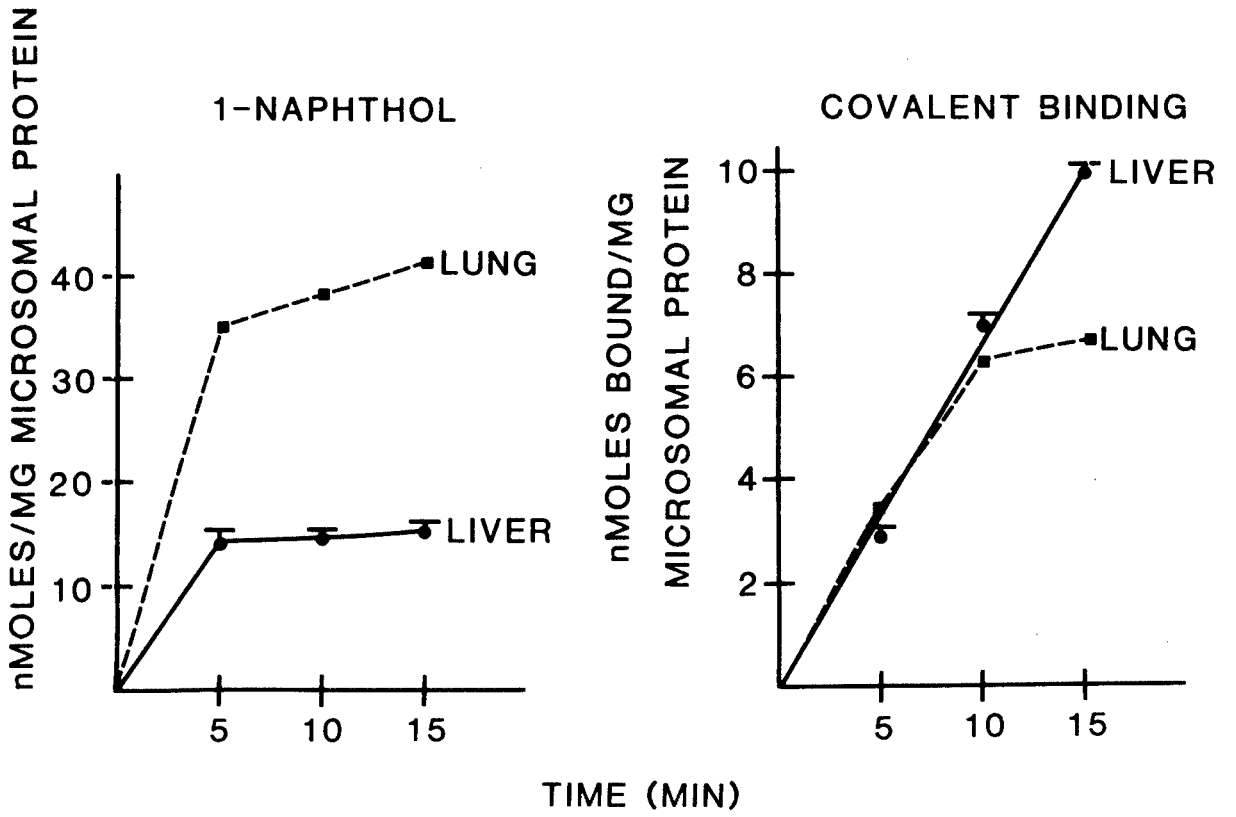



Figure 10. Time course metabolism of naphthalene to 1-naphthol and to covalently bound metabolites in mouse lung and liver microsomal preparations. Values are the means from 2 (lung) or 3 (liver) incubations containing: 0.1 mM $^{14}$C-naphthalene (2813 dpm/nmole), NADPH-generating system and 2 mg microsomal protein.

Rate of Formation of Covalently Bound Metabolites from 1-Naphthol in Lung and Liver Microsomal Incubations.

To be certain that the lack of difference in the rates of formation of covalently bound metabolites in lung vs liver was not simply due to the relative inability of lung microsomes to catalyze the formation of covalently bound metabolites from 1-naphthol, lung and liver microsomes were incubated with $^{14}$C-1-naphthol (0.1 mM, 886 DPM/nmole) and NADPH-generating system for periods of time from 5 to 20 minutes. As indicated in Figure 11, there was little difference in the rates of covalent binding of radioactivity from $^{14}$C-1-naphthol in lung vs liver microsomal incubations. Covalent binding in noncofactor control incubations was surprisingly high and indicated that the $^{14}$C-labelled substrate might have degraded radiochemically or that naphthol may be metabolized, in part, by non-NADPH requiring enzymes. The possibility that the substrate contained significant radiochemical impurities was tested by chromatographing an aliquot of the $^{14}$C-labelled 1-naphthol on a $C_{18}$ 3 μ Rainin (0.46 X 10 cm) column in 50% methanol/50% water at 0.8 mL/min. 1-Naphthol eluted at 10 min and was found to be 97.4% radiochemically pure.

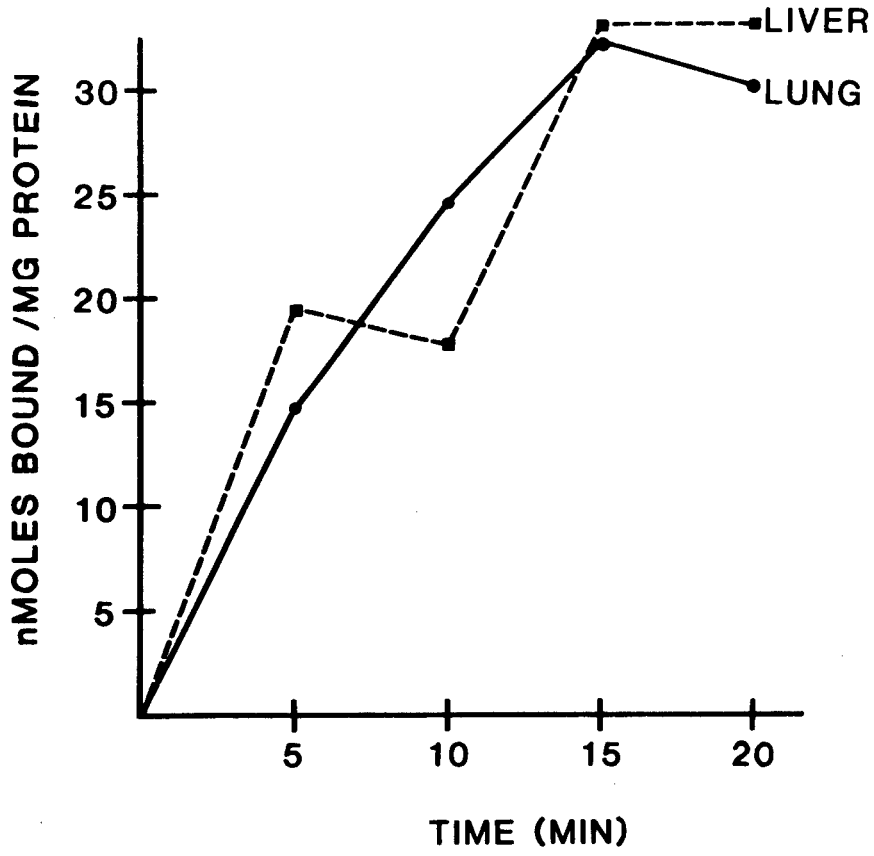



Figure 11. Time course formation of covalently bound metabolites from $^{14}$C-1-naphthol in mouse lung and liver microsomal incubations. Incubations contained in a total volume of 2 mL: $^{14}$C-1-naphthol (0.1 mM, 886 dpm/nmole), 2 mg microsomal protein and NADPH-generating system. Values obtained from incubations without NADPH have been subtracted.

Synthesis of $^{3}$H-Naphthalene -1,2-oxide.

To attempt to further define in quantitative terms the role of 1-naphthol vs naphthalene-1,2-oxide in the covalent binding observed in mice after naphthalene treatment, the synthesis of $^{3}$H-labelled naphthalene oxide has been attempted by the method of Dansette et al. (1974). All of the initial steps in the synthesis were carried out in a glove bag as required by Radiation Safety. 2-Tetralone (0.9 mL, 1.0 g) was reduced by the addition of successive amounts of 10 mg $NaBH_4$ followed by the addition of 25mCi $NaB^3H_4$ (150 mCi/mmole) and a further 66mg unlabelled sodium borohydride. Solvent was removed by evaporation under a stream of nitrogen and the remaining oil was dissolved in $CCl_4$. The $CCl_4$ solution was washed with 0.1 N HCl followed by 0.1 M $NaHCO_3$. The $CCl_4$ layer was placed in a 100 mL round bottomed flask fitted with a condensor. N-Bromosuccinamide (2.4g) and $\alpha,\alpha$-azoisobutyryldinitrile (10mg) were added and the mixture was heated at reflux (75$^{o}$ C) with a hot plate and heat lamp. The reaction mixture was allowed to cool and then was filtered into an incubation vial. This material was evaporated to dryness under nitrogen. The oily residue (155mg) was dissolved in 4 mL tetrahydrofuran and cooled on dry ice to -78$^{o}$ C. Sodium methoxide (387 mg in 4 mL THF) was added and the mixture was stirred 16 hours at room temperature. The reaction mixture was poured into 50 mL ice cold ether, and the ether phase was washed with 25 mL water followed by 25 mL 0.01M KOH. The ether phase was dried over $K_2CO_3$. An aliquot of the ether phase was spotted on a TLC plate (silica gel GF, 250 $\mu$) (origin prespotted with triethylamine), and the plate

was run 15 cm in benzene/chloroform/ethyl acetate/triethylamine 1:1:1:0.05. Sections (0.5cm) of the plate were scraped into liquid scintillation vials, 1 mL water and 3 mL Beckman EP scintillation fluid were added and the samples were counted in a gel phase after dark adaptation. Approximately 8% of the total radioactivity scraped from the plate cochromatographed with authentic 1-naphthol (Rf 0.33) and 89% of the total radioactivity scraped from the plate cochromatographed with authentic (cold) naphthalene oxide. Thus, to remove traces of 1-naphthol, the ether phase containing the synthetic product was evaporated to near dryness and was spotted on a TLC plate and chromatographed as before. The band cochromatographing with authentic naphthalene oxide was scraped from the plate, eluted with ether and the purity was rechecked by TLC. In this case the compound was homogeneous by TLC. To determine the specific activity, a sample of the radioactive oxide was scanned in the Cary spectrophotometer from 320 nm to 240 nm. The UV spectrum was inconsistent with the presence of the oxide. Moreover, addition of glacial acetic acid to the product resulted in only partial rearrangement to 1-naphthol. Several different solvent systems were used in an attempt to obtain separation of naphthalene oxide from the contaminating product on silica gel TLC plates and these were unsuccessful. Two products, containing approximately equal amounts of radioactivity, have recently been separated from the crude reaction mixture by chromatography on Whatman RP18 plates using ethanol/1% triethylamine as the eluting solvent. Upon acid treatment approximately 80% of one of these compounds rearranges to 1-naphthol-$^{3}$H. Additional tests will be needed to confirm the chemical nature and radiochemical purity of the product. This material will be incubated with microsomes in the presence and absence of NADPH-generating system with and without glutathione to determine whether naphthalene oxide is an intermediate in the covalent binding of radioactivity from labelled naphthalene.

**Metabolism of Naphthalene in Isolated Hepatocytes and Lung Cells**

HPLC Method for Measuring Oxidized and Reduced Glutathione, Cysteine and Glutathione Mixed Disulfides.

The method chosen for the determination of the cellular thiol status is that published by Reed and his associates (Reed et al., 1980; Farris and Reed, 1983) and involves derivatization of the free thiol with iodoacetic acid followed by the formation of a UV absorbing N-dinitrophenyl derivative with fluorodinitrobenzene. Initial studies have been done to establish this HPLC procedure in the laboratory and to determine the limit of sensitivity of this assay with the Waters M440 fixed wavelength UV detector (365 nm filter). One hundred and twenty-five microliters of solutions containing a total of 0.125, 0.250, 0.625, 1.25, 2.50, 5.0, or 7.5 nmoles GSH, GSSG and cysteine, and 1.25 nmoles gamma-glutamyl-glutamic acid (internal standard) were reacted with 12.5 µl iodoacetic acid (20 mg/ml), 112.5 µL 2 M KOH/2.4 M $KHCO_3$ followed 15 minutes later with 250 µl 1% ethanolic 1-fluoro-2,4-dinitrobenzene. Twenty-five to 100 µl aliquots of each solution were injected into a 25 cm 5 µ 3-aminopropyl column (CEL Associates, Houston, TX) run in 80% methanol/water (Solvent A) for 10 minutes at 1 mL/min. The solvent was then programmed to 0.8M sodium acetate/19% glacial acetic acid over 30 minutes. The following elution times were observed using these conditions: cysteine-31 minutes, gamma-glutamyl glutamic acid - 36 minutes, reduced glutathione-42 minutes and oxidized glutathione - 47.5 minutes. Peak height ratios of standard to internal standard were plotted vs the nmoles of thiol reacted (in the 125 µl initial sample). Injection of 100 µL of Standard 1 (125 pmoles) yielded peak heights (.005 AUFS) for cysteine, GSH and GSSG which were 15, 7.5 and 14% of full scale, respectively. These data indicate that quantities of thiol as low as 100 pmoles/sample could be quantitated and that reasonably precise quantitation may be achieved by this method (linear regression coefficients for the standard curves for GSH, GSSG and cysteine were 0.999, 0.989 and 0.995, respectively).

Hepatocyte Incubations

Methods have now been established in the laboratory for the isolation and maintenance of mouse liver hepatocytes with high viability. The procedure is as follows: male Swiss Webster mice (25-30 g, Charles River) are treated intravenously with heparin (200 i.u.) followed 30 min later by anesthetization with ether. Animal surgery is started between 10 and 11 a.m. to minimize interexperimental variations in glutathione levels. A cannula is placed in the inferior vena cava via the right atrium and the portal vein is severed. Retrograde perfusion is done for 5 min at 10 mL/min with $Ca^{++}$, $Mg^{++}$-free Hanks Buffer containing EGTA (0.5 mM) and HEPES (15 mM) at pH 7.4 and a temperature of 37° C (Buffer A). The perfusion buffer then is changed to $Mg^{++}$-free Hanks buffer, pH 7.4 containing 5 mM $CaCl_2$, 110 units collagenase/mL (Type IV, Sigma Chemical Co., St. Louis) and 15 mM HEPES maintained at a temperature of 37° C (Buffer B). Ten to 12 min after beginning perfusion with buffer B, the liver is excised, the gall bladder is removed and the liver is placed in a beaker containing buffer B. The hepatocytes are gently dispersed with forceps and the contents of the beaker are filtered through cotton gauze. After allowing the cells to settle for 5 min on ice, they are resuspended in Leibowitz's L-15 medium supplemented with 10% heat inactivated fetal calf serum, 15 mM HEPES and gentamycin (10 μg/mL) at pH 7.4. The cells are then layered on 36% Percoll in Hanks buffer (saturated with 95% $O_2$/5% $CO_2$) in centrifuge tubes and the tubes are centrifuged at 4° C in a Beckman SW 28 rotor at 20,000 x g for 20 min. The bottom layer, containing viable hepatocytes, is removed and centrifuged at 2000 x g for 1 min. The pellet, containing viable cells, is resuspended in the supplemented Liebowitz's medium and 50 μL are added to 150 μL of 0.4% trypan blue for cell counting and determination of viability. The viability of cells isolated by this procedure has been greater than 96% as assessed by trypan blue exclusion.

Several experiments have been done in an effort to establish the appropriate conditions for the quantitative estimation of the naphthalene oxide that can be generated within the hepatocyte and trapped as glutathione adducts on the exterior of the cell. The first three experiments were done using both $^{14}$C-naphthalene and $^{3}$H-glutathione in the same incubation. In the first experiments, concentrations of naphthalene of 0.5 mM were too high, and significant losses of viability were noted. The ability to estimate the amount of naphthalene oxide crossing the hepatocyte membrane will depend greatly on maintenance of intact cellular membranes. Thus, in the experiments to be reported here, mouse liver hepatocytes ($2 \times 10^6$ cells/ml) were incubated in the presence of 0.05 mM naphthalene (2071 dpm/nmole) and 0.5 mM glutathione with 20 CDNB units of glutathione transferase activity. Another flask containing hepatocytes, unlabelled naphthalene, glutathione transferases, and $^{3}$H-glutathione (0.5 mM, 14,900 dpm/nmole) was run simultaneously. The data, shown in Table 19 and Table 20, indicate that the dihydrodiol and all three glutathione adducts are formed during the incubation. Assuming that there are about $150 \times 10^6$ cells/mouse liver and about 25 mg microsomal protein, the rates of polar naphthalene metabolite formation (dihydrodiol plus conjugates) are roughly equivalent in isolated cell incubations and microsomal incubations. As a percentage of the total, dihydrodiol is higher in hepatocytes probably because of the large quantities of glutathione transferases used in the microsomal studies. The data in Table 19 also indicate that the ratio of rates of formation of adduct 2 to 1 and 2 to 3 in hepatocytes is similar to that found in liver microsomal incubations. As can be seen in Table 20 significant quantities of the naphthalene glutathione adduct contained $^{3}$H which would have to arise from the efflux of naphthalene oxide generated inside the cell and conjugation with $^{3}$H-GSH on the outside. The ratios of adducts formed is also quite different when the adducts are quantitated on the basis of $^{3}$H as compared with $^{14}$C. It should be noted that the amount of conjugate 2 calculated from $^{3}$H-label (Table 20) exceeds the total amount of conjugate 2 calculated from the $^{14}$C-label (Table 19). Possibly a contaminating peak is eluting along with conjugate 2.

As noted in Table 21, there is a UV absorbing radioactive peak (called Peak B) eluting at 55 min. The amount of this material increases with time in the cell medium. Peak B may be one of

a number of naphthalene metabolites including the glucuronide conjugate, the cysteine, N-acetylcysteine and cysteinylglycine adducts of naphthalene. To determine if peak B is a product arising from the initial formation of the naphthalene glutathione adducts, cysteine and N-acetyl-cysteine conjugates were synthesized from naphthalene oxide (using the same method used to prepare the glutathione adducts) to determine if their retention times were identical to that of Peak B. The results in Table 21 indicate that the retention time of Peak B does not correspond with elution of any of the other sulfur metabolites. The possibility that peak B is the glucuronide of 1-naphthol will be tested by incubating the collected peak with $\beta$-glucuronidase in the presence and absence of saccaric acid-1,4-lactone to determine whether 1-naphthol can be liberated.

Table 19

Formation of $^{14}$C-Labelled Naphthalene Glutathione Adducts in Isolated Hepatocytes[a]

| Incubation Time | | nmoles/2 x $10^6$ cells | | | | |
|---|---|---|---|---|---|---|
| | | Diol | Conj 1 | Conj 2 | Conj 3 | Peak B |
| 15 min | cell medium | 2.32 | 1.51 | 2.56 | 1.36 | 2.87 |
| | cells | ND | 0.20 | 0.50 | 1.33 | 9.48 |
| 30 min | cell medium | 0.512 | 1.70 | 3.15 | 1.59 | 6.95 |
| | cells | ND | 0.154 | 0.060 | 1.13 | 5.65 |
| 1 hr | cell medium | ND | 1.74 | 3.54 | 1.80 | 9.91 |
| | cells | ND | ND | 0.097 | NC | 3.04 |
| 4 hr | cell medium | ND | 1.94 | 4.22 | 1.80 | 10.31 |
| | cells | ND | ND | 0.051 | 0.064 | 0.486 |

[a] Incubations were done in 25 ml septum stoppered Erlenmeyer flasks under an atmosphere of 95% $O_2$/5% $CO_2$. In a total volume of 4 mL, flasks contained $^{14}$C-naphthalene (0.05 mM, 2071 DPM/nmole), GSH (0.5 mM), 20 CDNB units of glutathione transferase activity and 8 x $10^6$ cells. Medium was separated from cells by centrifugation and the dihydrodiol and naphthalene conjugates were separated by HPLC and quantified by counting metabolite fractions.

Table 20

Formation of $^{3}H$-Labelled Glutathione Naphthalene Adducts in Isolated Hepatocytes.[a]

| Incubation Time | | Nmoles/2 x $10^6$ cells Dihydrodiol | Conj 1 | Conj 2 | Conj 3 |
|---|---|---|---|---|---|
| 15 min | cell medium | 0.717 | 1.85 | 29.7 | 1.35 |
| | cells | ND | ---- | 1.02 | 0.11 |
| 30 min | cell medium | 0.02 | 2.90 | 22.0 | 1.12 |
| | cells | ND | ND | 0.60 | 0.05 |
| 1 hr | cell medium | ND | 1.97 | 16.9 | 0.72 |
| | cells | ND | ND | 0.42 | 0.02 |
| 4 hr | cell medium | ND | 1.4 | 17.0 | 0.85 |
| | cells | ND | ND | ND | ND |

[a]Incubation conditions were identical to those in Table 15 with the exception that unlabelled naphthalene (0.05 mM) and $^{3}H$-GSH 0.5 mM, 14900 DPM/nmole were used as substrates.
-- = Not determined
ND = Not detected

Table 21

HPLC Elution Times of Peak B and the Putative Cysteine-Naphthalene Adduct and N-Acetylcysteine-Naphthalene Adduct[a]

| Compound | Elution Time | Comments |
|---|---|---|
| Peak B | 55 min | |
| N-acetyl-cysteine-naphthalene | 49 min | |
| Cysteine-naphthalene | 11 & 11 1/2 min | 2 large UV peaks |
| | 12 1/2 min | 1 small peak |

[a] Chromatography was performed on a 5 μ $C_{18}$ Altex column (0.46 x 15 cm) using a mobile phase of 5% acetonitrile, 1% acetic acid, and $H_2O$ at a flow rate of 1.0 mL/min.

In an attempt to further support the view that naphthalene oxide formed intracellularly is capable of effluxing from intact hepatocytes, the experiment cited above was repeated. The initial cell viability of mouse hepatocytes isolated by collagenase perfusion was brought to 97% by centrifugation on a Percoll density gradient. In a total volume of 1.5 mL, incubations contained: 7.5 CDNB units of glutathione transferase activity, 1.5 million cells, and either $^{14}C$-naphthalene at 0.05 mM or 0.5 mM, 2144 dpm/nmole or $^{3}H$-glutathione 0.5 mM, 1570 dpm/nmole. Incubations were done in Hanks buffer with 20 mM HEPES at a pH of 7.4. Incubations containing labelled naphthalene had unlabelled glutathione and vice versa. Following an incubation at 37° C for 7, 15, or 30 min, the vessels were allowed to cool on ice. Cells were removed from the medium by centrifugation and 1 mL methanol was added to the medium. The cells were resuspended in 1 mL Hanks buffer and were again collected by centrifugation. One mL

methanol was added to the cells to precipitate the protein, and naphthalene dihydrodiol and the three glutathione adducts were quantitated in the aqueous extract in the usual fashion. Viability as assessed by trypan blue exclusion dropped from 97% initially to 85% in cells incubated in the presence of 0.05 mM naphthalene and to 53% in those incubations containing 0.5 mM naphthalene at 30 min.

The data in Table 22 indicate that, in contrast to the results obtained in incubations of hepatic microsomes with semipurified glutathione transferases, dihydrodiol formation predominates over the formation of the three glutathione conjugates from naphthalene in incubations with isolated hepatocytes. The major portion of the water-soluble metabolites from naphthalene are found extracellularly at all time points during the incubation, and the formation of these metabolites was reasonably linear with time in the incubations containing 0.5 mM naphthalene. The data from this experiment provide further support to the view that naphthalene oxide formed intracellularly is capable of effluxing from hepatocytes and forming glutathione conjugates extracellularly. This appeared to be true at both the 0.05 and 0.5 mM naphthalene concentrations, and thus the data may indicate that substantial depletion of glutathione is not necessary before these metabolites can leave the cell. As noted earlier in this discussion, however, there was a marked decrease in cell viability at the high concentration of naphthalene and thus the data at this concentration must be interpreted cautiously. However, at the 0.05 mM naphthalene concentration, viability remained acceptable over the 30 min time period and there was a significant amount of tritium labelled conjugate observed in the cell medium at the 15 min time point. These studies will have to be repeated using additional concentrations of naphthalene but the initial data do indicate that naphthalene oxide formed by enzymes localized on the endoplasmic reticulum is capable of escaping attack by glutathione and the soluble glutathione transferases. Future studies are planned to assess the half life of naphthalene oxide in the incubation medium and compare this with the half life of this oxirane metabolite formed by isolated hepatocytes to determine whether the oxide could be extruded by the cell in a lipid or lipoprotein envelope. Such a phenomenon would, in part, explain why very high doses of naphthalene oxide had to be administered before significant glutathione depletion was observed in vivo.

Table 22

Formation of Radiolabelled Glutathione Conjugates in Hepatocyte Incubations Containing Either $^{14}$C-Naphthalene or $^{3}$H-Glutathione[a]

| Substrate NAPH | Substrate GSH | Sample | Time | Diol | Conj 1 | Conj 2 | Conj 3 |
|---|---|---|---|---|---|---|---|
| | | | | | Nmoles/Million Cells | | |
| 0.05* | 0.5 | Medium | 7 | 2.04 | 0.98 | 0.76 | 1.13 |
| 0.05 | 0.5* | Medium | 7 | ---- | 0.61 | 1.08 | 1.90 |
| 0.05* | 0.5 | Medium | 15 | | ---------Data Not Available----- | | |
| 0.05 | 0.5* | Medium | 15 | ---- | 2.49 | 3.01 | 2.51 |
| 0.05* | 0.5 | Medium | 30 | 3.22 | 3.31 | 4.88 | 2.46 |
| 0.05 | 0.5* | Medium | 30 | | ---------Data Not Available----- | | |
| 0.5* | 0.5 | Medium | 7 | 4.01 | 1.77 | 1.00 | 1.82 |
| 0.5 | 0.5* | Medium | 7 | ---- | 1.10 | 0.86 | 1.51 |
| 0.5* | 0.5 | Medium | 15 | 7.17 | 3.75 | 2.37 | 3.49 |
| 0.5 | 0.5* | Medium | 15 | ---- | 2.86 | 2.36 | 3.14 |
| 0.5* | 0.5 | Medium | 30 | 18.0 | 7.78 | 5.10 | 7.14 |
| 0.5 | 0.5* | Medium | 30 | ---- | 5.16 | 5.83 | 6.11 |
| 0.05* | 0.5 | Cells | 7 | 0.13 | 0.53 | 0.32 | 0.52 |
| 0.05 | 0.5* | Cells | 7 | | ---------------Not Done--------- | | |
| 0.05* | 0.5 | Cells | 15 | 0.10 | 0.43 | 0.61 | 0.35 |
| 0.05 | 0.5* | Cells | 15 | | ---------------Not Done--------- | | |
| 0.05* | 0.5 | Cells | 30 | 0.03 | 0.14 | 0.26 | 0.18 |
| 0.05 | O.5* | Cells | 30 | | ---------------Not Done--------- | | |
| 0.5* | 0.5 | Cells | 7 | 0.24 | 0.85 | 0.33 | 0.74 |
| 0.5 | 0.5* | Cells | 7 | | ------------Not Detected-------- | | |
| 0.5* | 0.5 | Cells | 15 | 0.21 | 0.84 | 0.37 | 0.62 |
| 0.5 | 0.5* | Cells | 15 | | ------------Not Detected-------- | | |
| 0.5* | 0.5 | Cells | 30 | 0.51 | 0.84 | 0.36 | 0.58 |
| 0.5 | 0.5* | Cells | 30 | | ------------Not Detected-------- | | |

[a]Hepatocytes were incubated at a concentration of 1 x $10^6$ cells/mL, with 0.5 or 0.05 mM naphthalene (*-2114 dpm/nmole), 7.5 CDNB units glutathione transferase activity, and 0.5 mM glutathione (*-1570 dpm/nmole). Sufficient material was present for only one analysis of conjugate formation in the cell medium; in several cases good quantitative data could not be obtained. Significant tritium counts (>10 cpm above background) were not observed in glutathione adducts isolated from cell fractions of incubations done in the presence of 0.5 mM unlabelled naphthalene with 0.5 mM $^{3}$H-glutathione.

**Intravenous Administration of Potentially Toxic Metabolites of Naphthalene**

Following the advice of the Scientific Advisory Board, we have begun a project to examine the toxicity of potential naphthalene metabolities after intravenous administration. While injection of these compounds as a bolus will not mimic the situation which could occur in vivo, namely the formation and slow release of such metabolites from the liver, it could give us information on candidates which would likely be severely cytotoxic and could suggest which compounds should be administered at μ l/hour rates i.v. with a mini pump. Recent studies with bromobenzene and a number of the reactive pyrollizidine alkaloid derivatives do support this approach. (Lau et al. 1983; Mattocks and Driver, 1983; Rush et al. 1983).

Intravenous administration of 1,4-naphthoquinone

Initially, the following compounds will be administered to at least 5 animals at each of at least 4 dose levels: 1,2-naphthoquinone, 1,4-napthoquinone, 1,2-dihydroxy-1,2-dihydronaphthalene, naphthalene, 1-naphthol, naphthalene-1,2-oxide, naphthalene-1,2,3,4-diepoxide and 1,2-dihydroxy-1,2-dihydro-3,4-epoxynaphthalene. All compounds will be recrystallized before use and their purity will be checked by melting point (where possible), HPLC, or TLC. Epoxide derivatives of naphthalene will be submitted for NMR for structural verification. The choice of solvents is limited since a solvent must be chosen in which most of the derivatives will be readily soluble yet which has relatively low toxicity. The use of ethanol was investigated since this is a solvent in which naphthalene-1,2-oxide is both readily soluble and stable. To determine whether ethanol administered i.v. resulted in any observable bronchiolar toxicity, groups of 2 mice each were given absolute ethanol (reagent grade) at doses of 1, 2, 3 and 4 μL/gram body weight. Animals were sacrificed 24 hours after vehicle administration and the lung was removed, fixed in Karnovsky's fixative and processed for light microscopic examination. Pulmonary injury was not observed in any of the treatment groups (Table 23). Because large amounts of 1,4-naphthoquinone could not be dissolved in ethanol, dimethyl-formamide was used as a vehicle for administration. Intravenous administration of dimethylformamide at a dose of 2 μL/g resulted in some disruption of the cuboidal arrangement of the bronchiolar epithelium. In some airways, the characteristic protusions of Clara cells were absent.

1,4-Naphthoquinone (Aldrich) was recrystallized from ethanol to yield medium brown crystals mp 122-123°. The recrystallized quinone was dissolved in DMF and administered at 200, 50, 25, and 12.5 mg/kg i.v. Animals treated with 200 mg/kg died before the end of the injection. 1,4-Napthoquinone administered i.v. at doses of 25 and 50 mg/kg caused 100% lethality within 30 min of the injection (Table 23). Blood was noted exiting the mouth and nose of all treated animals, and examination of the lungs revealed massive pulmonary hemorrhage. At 12.5 mg/kg, all three animals survived 24 hours. Moderate bronchiolar injury was noted in one of the three treated animals with vacuolization of many bronchiolar epithelial cells. This experiment will need to be repeated using larger numbers of animals and a different solvent. 1,2-Napthoquinone, a known metabolite of naphthalene, will also need to be tested. A good recrystallization of the crude material (90% Aldrich) could not be achieved and thus the compound will be synthesized from 1-amino-2-naphthol hydrochloride (Fieser and Fieser, 1967). This synthesis yields a product which is very nearly pure.

Table 23

Toxicities of Administrative Vehicles and 1,4-Naphthoquinone Administered Intravenously.[a]

| Vehicle | Compound/Dose[b] | n | % mortality | Time of Death After Inj | Severity of Pulm. Lesion 0 | + | ++ | +++ |
|---|---|---|---|---|---|---|---|---|
| ethanol 100% | 1 µL/g | 2 | 0 | -- | 2 | | | |
| | 2 µL/g | 2 | 0 | -- | 2 | | | |
| | 3 µL/g | 2 | 0 | -- | 2 | | | |
| | 4 µL/g | 2 | 0 | -- | 2 | | | |
| Dimethyl-formamide | --2 µL/g | 4 | 0 | -- | 4 | | | |
| DMF | 1,4-naphthoquinone | | | | | | | |
| | 200mg/kg-2 µL/g | 3 | 100 | -- | | | | |
| | 50mg/kg-0.5 µL/g | 5 | 100 | 30 min | | | | |
| | 25mg/kg-0.5 µL/g | 3 | 100 | 30 min | | | | |
| | 12.5mg/kg-.5 µL/g | 3 | 0 | -- | 2 | 1 | | |

[a] Male Swiss Webster mice (23-29 g) were treated intravenously at the indicated dose and were sacrificed 24 hours later by pentobarbital overdose. The trachea was cannulated and the lungs were inflated with Karnovsky's fixative. Paraffin sections (5-6 µm) were stained with hematoxylin and eosin and examined by light microscopy.

[b] Dose for the vehicle is given in µL/g body weight and the dose of toxicant administered is recorded in mg/kg.

[c] Pulmonary bronchiolar lesions were graded as mild (+) denoting disruption of the normal cuboidal appearance of the bronchiolar epithelium with swelling but no exfoliation of bronchiolar epithelial cells. This category includes pulmonary epithelium with pyknotic nuclei and lightly stained cytoplasm. Lung lesions graded as moderate (++) include lungs where cellular vacuolization and some exfoliation and sloughing of bronchiolar epithelial cells is noted. Severe (+++) pulmonary bronchiolar damage denotes lungs in which there is extensive exfoliation and sloughing of cells into the airway lumen.

Effect of Intravenous Administration of Naphthalene Oxide on Pulmonary, Hepatic, and Renal Nonprotein Sulfhydryl Levels.

The first experiment with naphthalene oxide was designed to determine whether this compound is capable of surviving in the bloodstream for a sufficient period of time to reach lung, liver, or kidney, and, if so, whether the compound is capable of crossing cell membranes. A method for doing this is to determine whether reduced sulfhydryl content of the tissues is depleted after intravenous administration of naphthalene oxide.

In the first experiment, naphthalene oxide, dissolved in ethanol, was administered intravenously over a period of approximately 30 sec at doses of 32, 48, or 64 mg/kg to groups of 1 to 5 male mice (Swiss Webster, Charles River, 21-24 g). Control mice received ethanol only.

Because substantial decreases in tissue glutathione levels were not noted in this first experiment, a second batch of naphthalene oxide was prepared and doses of 50, 100, 200, and 400 mg/kg were administered. Ninety minutes after administration, the mice were killed by cervical dislocation, and lungs, live, and kidney were removed and rapidly frozen. Tissue reduced sulfhydryl levels were determined by the colorimetric procedure of Ellman (1959).

The data in Figure 12 indicate that naphthalene oxide administered intravenously produces a dose-dependent depletion of pulmonary glutathione levels without any significant effect on hepatic or renal levels. The depletion of pulmonary glutathione only becomes significant at the 75 mg/kg dose (Students two-tailed T test, $p < .05$), a dose that seems unrealistically high if one is to propose that naphthalene oxide, effluxing from the hepatocyte, results in part of the depletion of pulmonary glutathione caused by intraperitoneal administration of high doses of naphthalene. The preparation of naphthalene oxide used in this experiment was checked by TLC immediately before and after administration to the animals and there was no detectable breakdown to 1-naphthol over the course of the experiment, and thus degradation of the compound cannot account for the observed lack of effect at low doses. There are a number of things which must be considered when discussing experiments such as these. At present we do not know what the half life of naphthalene oxide is in the blood. It is possible that under in vivo conditions small amounts of naphthalene oxide exiting the liver are stabilized (perhaps by lipoproteins) in the blood and can reach the lungs more effectively than the large bolus dose administered in these experiments. Lau et al.(1983) have recently reported levels of bromobenzene-3,4-oxide in the blood of 2.55 nmoles/mL and suggest that the formation of this oxide by the liver may have a role in bromobenzene-induced extrahepatic toxicity in the glutathione-depleted animal. This is going to be very difficult to determine satisfactorily. In future experiments, it will be important to determine the half-life of naphthalene oxide in blood and determine precisely how much, and at what concentration, naphthalene oxide effluxes from intact hepatocytes.

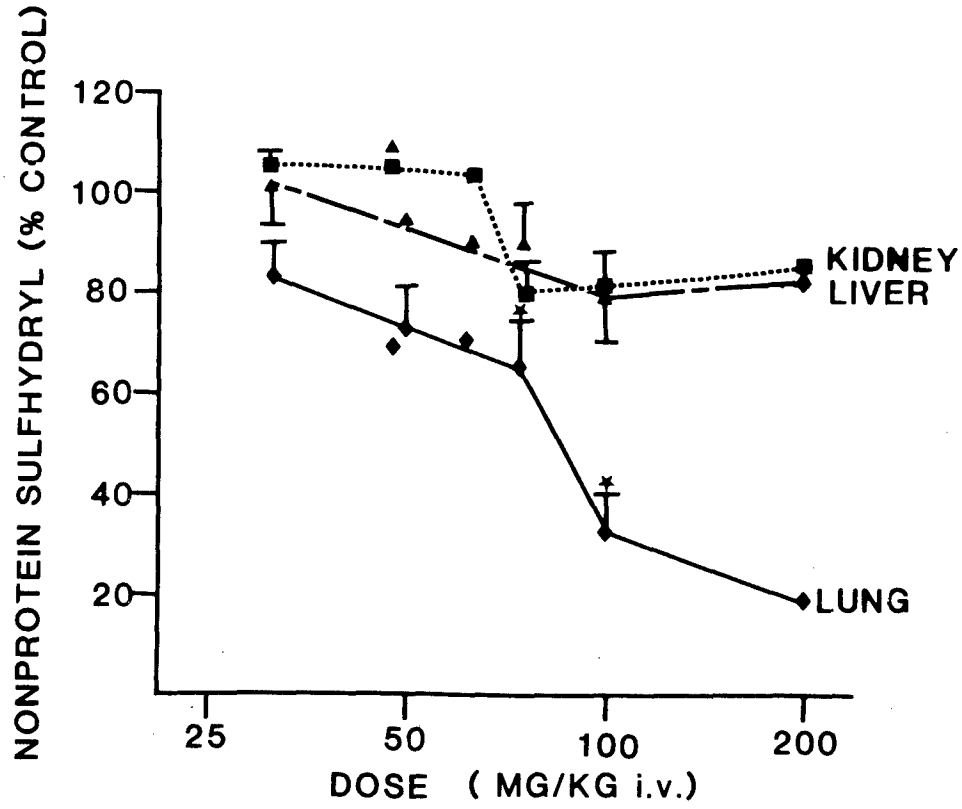



Figure 12. Hepatic, pulmonary, and renal nonprotein sulfhydryl levels 90 min after the administration of naphthalene oxide i.v.. Values represent the mean of two or the mean ±S.E. for at least three animals per data point. Control nonprotein sulfhydryl levels were 0.401±0.10, 2.64±0.15 and 1.40±0.10 mg/g for lung, liver and kidney, respectively (mean±S.E., n=5).

Our intention is to pursue the question of efflux and circulation of naphthalene oxide further. It will be important to determine how much, if any, naphthalene oxide can be found in the blood after i.p. administration of naphthalene. As described in the following section, this potentially can be done either by the method used by Lau and her associates (1983) (that is, incubating blood with $^{3}$H-GSH and glutathione transferases followed by quantitation of the GSH adducts) or by chemical reaction with 3,4-dichlorobenzenethiol (Preussmann et al., 1976; Muller et al., 1978). The possible disadvantage of the approach of measuring glutathione adducts may be that the adducts are not formed before partial rearrangement of the naphthalene oxide to naphthol has occurred. From studies presented in the section on Incubation of Naphthalene Oxide with $^{3}$H-GSH, this does not appear to be a problem since only trace quantities of naphthol could be detected in the incubations containing glutathione and glutathione transferases.

Attempt to Demonstrate the Presence of Naphthalene Oxide in the Blood In Vivo.

This experiment was designed to determine if any naphthalene oxide could be detected in mouse blood after intraperitoneal administration of napthalene. Four male Swiss Webster mice (29-38g) were given 880 mg/kg buthionine sulfoximine (0.1 mL/10g bw in saline, subcutaneously) to deplete hepatic glutathione. Two hours later, naphthalene (400 mg/kg, dissolved in corn oil) was administered intraperitoneally. Forty-five or 60 minutes after naphthalene administration, 2

animals were sacrificed by lethal injection of pentobarbital, blood was removed by cardiac puncture and placed in an incubation flask containing $^{3}$H-glutathione (12,500 dpm/nmole, 0.1mM) and 10 CDNB units of glutathione transferase activity. These samples were incubated at 37$^{0}$ C for 15 min, 2 volumes of ice-cold methanol were added to precipitate the protein and the samples were centrifuged for 30 min. Two mL of the remaining supernatant was taken to dryness on the evaporator, and this was reconstituted in 200 µl water. One hundred microliters was chromatographed under the conditions used routinely for chromatography of the glutathione conjugates and 1 mL samples of the column eluate were collected and counted. Samples from the blood taken at 45 min post naphthalene failed to show any detectable radioactivity eluting with the glutathione conjugate peaks. However, the elution profile prepared from the incubations of blood taken at 1 hour showed a small peak of tritium counts eluting just after the peak of UV absorbance corresponding to conjugate 2. (There is about a 30-60 sec delay caused by the length of tubing leading from the UV bench to the fraction collector) (Figure 13). This peak represents roughly 1000 dpm. Even though the starting substrate was 12,500 dpm/nmole, erythrocytes contain large amounts of glutathione—reported to be on the order of 1.25 mM in mice (Chasseaud, 1979). Thus, the final specific activity would be approximately 400 dpm/nmole and the peak coeluting with conjugate 2 represents on the order of 1 nmole of material. This observation is preliminary and requires confirmation by another means.

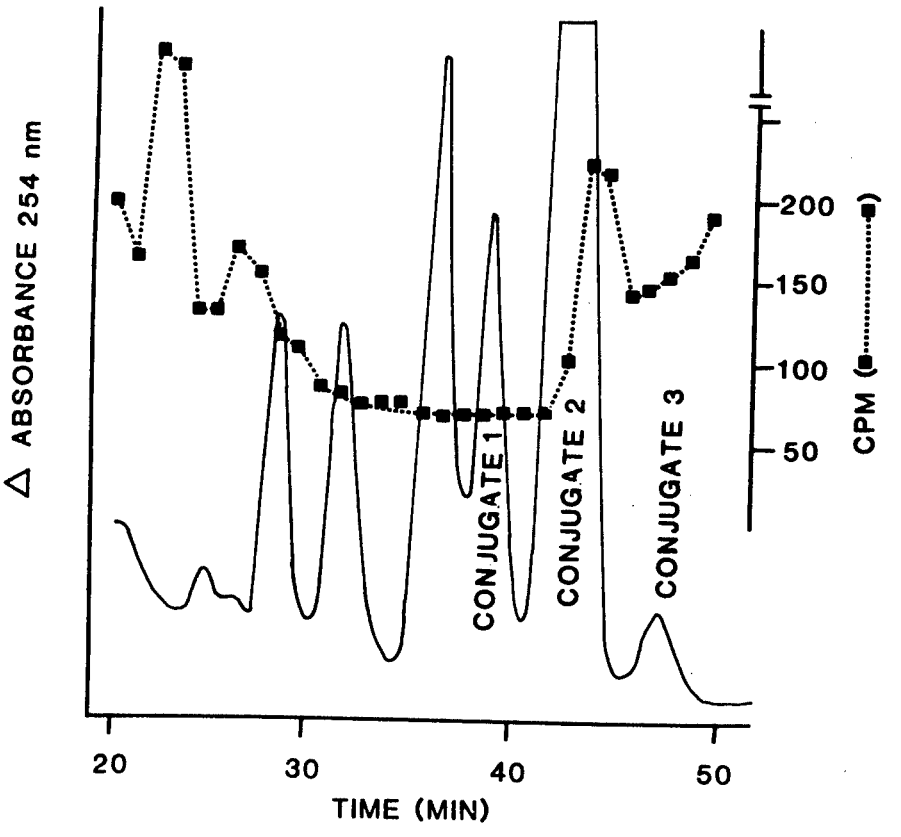



Figure 13. Radioactive and UV elution profile of an extract from an incubation of $^{3}$H-glutathione, glutathione S-transferases (10 CDNB units, affinity column purified), and blood taken by cardiac puncture from 2 mice treated 4 hours earlier with buthionine sulfoximine (880 mg/kg) and 2 hours earlier with naphthalene (400 mg/kg). Fractions of the column eluate were collected at 1 min intervals and counted for 20 min.

**Use of Cobalt Protoporphyrin as a Potent and Long-Lasting Inhibitor of Cytochrome P450 Monooxygenase Mediated Xenobiotic Metabolism**

Effect of Cobalt Protopropyrin on the In Vitro Metabolism of Naphthalene

Drummond and Kappas (1982) recently reported prolonged and substantial depletion of rat liver cytochrome P450 monooxygenases after a single subcutaneous dose of cobalt protoporphyrin (125 μ moles/kg). This compound is a potent inhibitor of α-amino levulinate synthetase (the rate limiting step in heme biosynthesis) and an inducer of heme oxygenase and may offer advantages over the classic cytochrome P450 inhibitors, piperonyl butoxide or SKF 525A. These latter inhibitors require metabolism by the monooxygenase enzymes and are capable, in multidose studies, of inducing the P450 enzymes. Because of a different mechanism of action, these drawbacks are not associated with the use of cobalt protoporphyrin. More importantly, however, was the possibility that cobalt protoporphyrin could differentially decrease liver but not lung P450. Drummond and Kappas state that the induction of renal heme oxygenase by cobalt protoporphyrin occurs 6 to 10 days after administration while maximal induction of the liver enzyme occurs almost immediately. The ability to differentially decrease hepatic cytochrome P450 in vivo would provide a means for examining the role of hepatic metabolism in extrahepatic organ toxicities (i.e., lung and kidney) caused by agents such as naphthalene or the chlorinated ethylenes, vinyl chloride and trichloroethylene. Thus, a series of studies have been done to explore the possibility that cobalt protoporphyrin could be used as a tissue-selective inhibitor of P450 monooxygenase activity.

The first experiment was designed to determine whether cobalt protoporphyrin administration was capable of altering the metabolism of naphthalene in vivo. Cobalt protoporphyrin (Porphyrin products, Logan, Utah) was dissolved in 0.5 M NaOH, 0.1 M HCl was added to bring the solution to neutrality, and sufficient isotonic saline was added so that 0.1 mL dose solution was administered subcutaneously per 10 g body weight. Groups of animals were treated with cobalt protoporphyrin (125 μ moles/kg) 1, 2, 3, 4, and 7 days before sacrifice. A control group (23 animals) was given saline s.c. 24 hours before killing. A lobe of lung and liver from each of the first five animals of each group was removed and frozen for glutathione analysis. Previous studies have shown that cobaltous chloride, a potent depletor of cytochrome P450, produces a marked increase in reduced glutathione levels and thus cannot appropriately be used in studies to determine the potential roles of metabolically-generated reactive metabolites in tissue toxicity (Sasame and Boyd, 1978). Microsomes and cytosol were prepared from the remaining tissue by standard techniques.

The data in Figure 14 indicate that a single s.c. dose of 125 μ moles/kg cobalt protoporphyrin markedly decreased both pulmonary and hepatic microsome-catalyzed formation of covalently bound metabolites from naphthalene. In liver, this effect appeared to be greatest for the first two days after exposure; at later times microsomal activity appeared to recover slightly. In lung microsomes, the metabolism of naphthalene to reactive metabolites was not decreased to the extent noted in liver microsomal incubations during the first three days after cobalt protoporphyrin. However, after seven days, pulmonary and hepatic microsomal metabolizing activity was inhibited to a similar extent. Analysis of hepatic microsomal cytochrome P450 levels indicated that cobalt protoporphyrin resulted in an approximate 50% decrease in comparison to control (data not shown). Whether the differential inhibition noted on day 1 will be sufficient to allow determination of the relative roles of metabolism in liver and lung in the formation of pulmonary cytotoxic metabolites will require further study.

In an attempt to increase the selectivity of response to cobalt protoporphyrin, groups of 18 mice each were treated with either saline or cobalt protoporphyrin intraperitoneally. Animals were killed 24 hours later and microsomes were prepared from lung and liver. Cobalt protoporphyrin (125 μ moles/kg) resulted in significant mortality (39%) and in substantial

decreases in pulmonary and hepatic microsomal metabolism of naphthalene to reactive metabolites. The data in Figure 14 indicate that i.p. administration of cobalt protoporphyrin results in a less selective decrease in hepatic compared to pulmonary monooxygenase activity (Figure 14, data shown in circles).

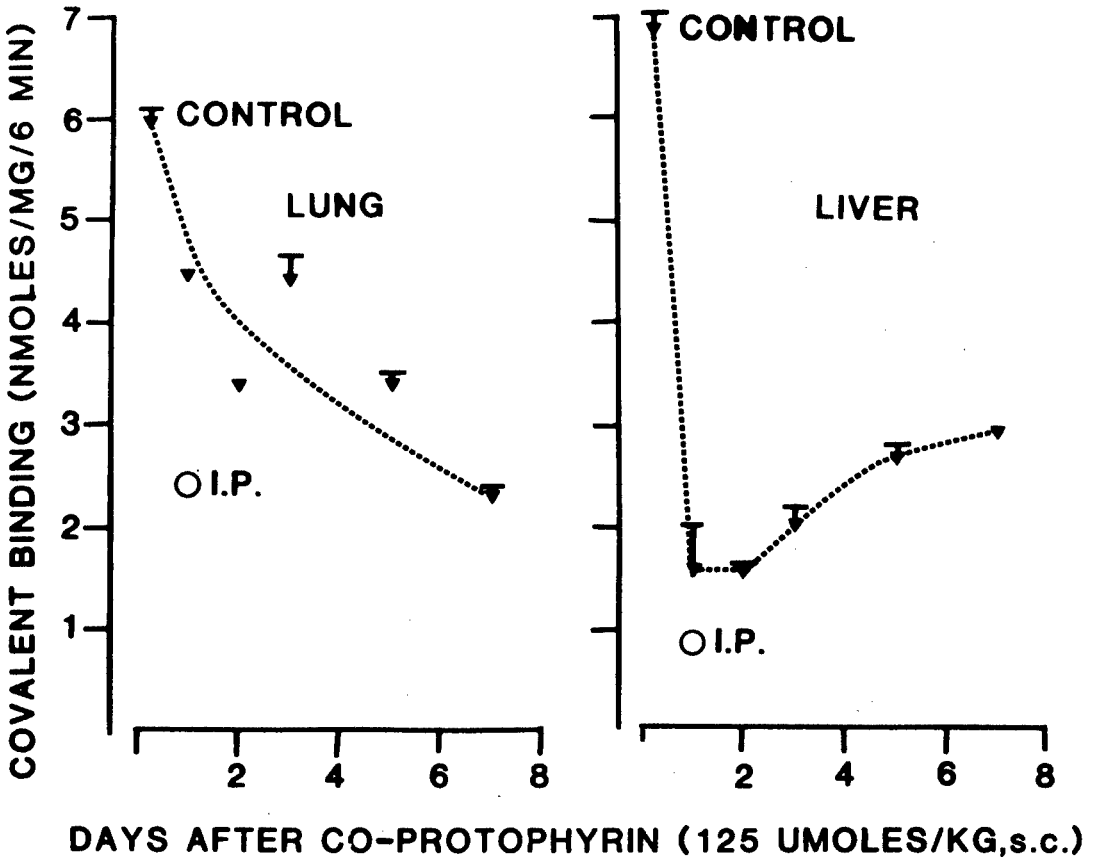



Figure 14. Effect of cobalt protoporphyrin on hepatic and pulmonary monooxygenase activity. Incubations consisted of microsomes (2 mg), NADPH-generating system and $^{14}$C-naphthalene (1mM, 1232 dpm/nmole) in a total volume of 2 mL. Incubations were for 6 min at 37°C and covalent binding was determined in the precipitated protein as described previously. Values are the mean of 2 or mean ± S.E. for three incubations.

Effect of Cobalt Protoporphyrin Treatment on Tissue Reduced Glutathione Levels

Pulmonary and hepatic glutathione levels in the lobe of liver and lung removed from control and treated animals indicated that cobalt protoporphyrin produced a slight but significant increase in pulmonary glutathione levels on days 2-7 and a substantial decrease in hepatic glutathione levels on days 1 through 7 (Figure 15). Because the effect of cobalt protoporphyrin appeared to be sustained for a substantial period of time, the experiment was repeated. Hepatic glutathione levels dropped to approximately 50% of control on days 1 through 5 and returned to control levels at day 7. Consistent with the previous studies, pulmonary reduced glutathione levels were slightly higher than control on days 2 and 3 after cobalt protoporphyrin.

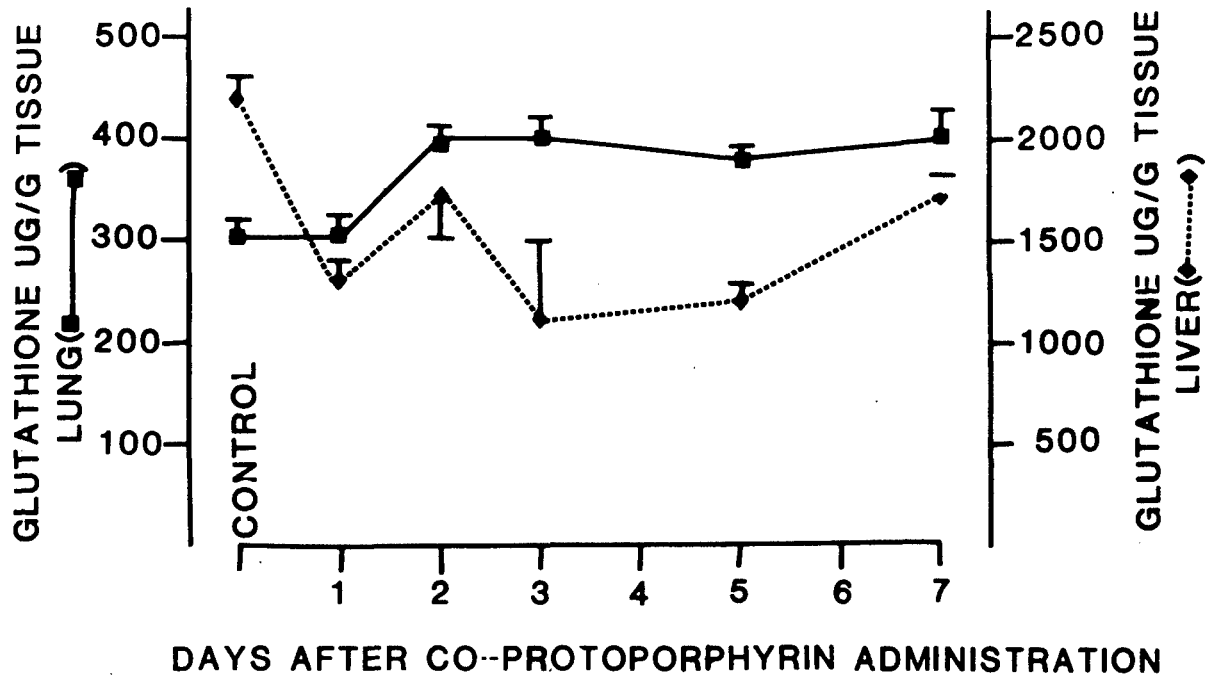



Figure 15. Effect of cobalt protoporphyrin on hepatic and pulmonary glutathione levels in the mouse. Cobalt protoporphyrin was administered at a dose of 125 μ moles/kg, s.c. and glutathione levels were assayed in tissues from 5 animals. Values are the mean ±S.E.

Effect of Cobalt Protoporphyrin on the Pulmonary Bronchiolar Necrosis by Naphthalene and 2-Methylnaphthalene

To determine whether pretreatment with cobalt protoporphyrin would provide protection against naphthalene or 2-methylnaphthalene-induced bronchiolar necrosis, groups of 5 animals each were treated with either saline (subcutaneously) or cobalt protoporphyrin (125 μ moles/kg, s.c.) followed 24 hours later by either corn oil or naphthalene (300 or 600 mg/kg i.p. in corn oil). All animals were killed by pentobarbital overdose 24 hours after toxicant administration and lungs were fixed in situ by tracheal infusion of Karnovsky's fixative. Histopathological sections (5-6 μ paraffin, H + E) were examined in a blind fashion, and lung sections were scored as 0 (no damage) + (mild swelling of bronchiolar epithelial cells but no sloughing), ++ (sloughing of cells in the terminal airways), +++ (sloughing of the bronchiolar and bronchial epithelium). The data in Table 24 indicate that pretreatment with cobalt protoporphyrin provides no protection against the bronchiolar damage by either naphthalene or 2-methylnaphthalene; in fact quite the opposite appears to be the case. There were no deaths in the saline and naphthalene 600 mg/kg treated animals, while there was a 60% mortality rate in the group pretreated with cobalt protoporphyrin. Likewise, the severity of bronchiolar lesions in the cobalt protoporphyrin plus 2-methylnaphthalene treated animals appears to be slightly greater than in the comparable saline pretreated groups. There are several plausible explanations for thess data. If the data showing that cobalt protoporphyrin treatment causes a sharp decrease in hepatic nonprotein sulfhydryl levels are correct, it is quite possible that far more unchanged naphthalene and 2-methylnaphthalene reach the lungs in cobalt protoporphyrin pretreated animals. Moreover, if

reactive metabolites circulating from the liver play a role in the pulmonary lesion, a decrease in glutathione levels, even with a decrease in P450, may be a factor in determining the overall extent of the pulmonary lesion. Overall, these studies indicate that cobalt protoporphyrin may not be an appropriate agent for determining the role of cytochrome P450-dependent metabolism in extrahepatic tissues.

Table 24

Effect of Cobalt Protoporphyrin Pretreatment on Naphthalene and 2-Methylnaphthalene Induced Pulmonary Bronchiolar Necrosis[a]

| Inhibitor Pretreatment | Toxicant Treatment | % Mortality (24 hrs.) | Severity of Lung Lesion 0 | + | ++ | +++ |
|---|---|---|---|---|---|---|
| saline | corn oil | 0 | 5/5 | | | |
| Co-Proto | corn oil | 0 | 5/5 | | | |
| saline | naphthalene 300 mg/kg | 0 | | | | 5/5 |
| Co-Proto | naphthalene 300 mg/kg | 0 | | | | 5/5 |
| saline | naphthalene 600 mg/kg | 0 | | | | 5/5 |
| Co-Proto | naphthalene 600 mg/kg | 60 | | | | 2/2 |
| saline | 2-MeNa 300 mg/kg | 0 | | 4/5 | 1/5 | |
| Co-Proto | 2-MeNa 300 mg/kg | 0 | | | 2/5 | 3/5 |
| saline | 2-MeNa 600 mg/kg | 0 | | | | 5/5 |
| Co-Proto | 2-MeNa 600 mg/kg | 0 | | | | 5/5 |

[a] Groups of 5 male Swiss Webster mice (Charles River) were treated subcutaneously with either saline or cobalt protoporphyrin (125 μ moles/kg) (10 mL/kg). Twenty-four hours later the animals received corn oil, naphthalene or 2-methylnaphthalene in corn oil (10 mg/kg) at the specified doses. Animals were sacrificed 24 hours after hydrocarbon administration.

**Arachidonic Acid-Dependent Metabolism of Naphthalene and 2-Methylnaphthalene**

Since the pulmonary Clara cell necrosis by 2-methylnaphthalene does not appear to be related to the cytochrome P450 monooxygenase-mediated metabolism of this compound and since the in vivo covalent binding after administration of 2-methylnaphthalene was not piperonyl butoxide inhibitable (Griffin et al., 1982), it seemed possible that other mechanisms for the oxidation of these hydrocarbons might be operative. In addition, studies with naphthalene have shown that the in vivo covalent binding of reactive metabolites to renal macromolecules is similar to liver at all doses and times studied; yet, kidney NADPH-dependent, microsome-catalyzed metabolism of naphthalene to intermediates which bind covalently to protein is marginally detectable. Although these data support the hypothesis that the intermediates which become bound covalently in the kidney originate in the liver, another possible explanation is that naphthalene is being metabolized via non-NADPH-dependent pathways in the kidney. Since a number of compounds, including benzo(a)pyrene, undergo oxidation during prostaglandin synthesis (Marnett et al., 1978; Zenser et al., 1978; Zenser et al., 1983), and because Sivarajah et al. (1983) have shown that prostaglandin synthetase-mediated metabolism of benzo(a)pyrene-7,8-diol to

tetrols occurs at approximately half the NADPH-dependent rate in Clara cells and twice the NADPH-dependent rate in Type II cells, the possibility that naphthalene and 2-methylnaphthalene are oxidized via arachidonate-dependent pathways has been examined.

Covalent binding of reactive naphthalene/2-methylnaphthalene metabolites to microsomal protein was assayed by standard procedures. The data in Table 25 indicate that high levels of NADPH-dependent covalent binding of naphthalene and 2-methylnaphthalene metabolites occurred in pulmonary and hepatic but not renal microsomal incubations. These data confirm the results of earlier studies. Detectable arachidonic acid-supported metabolism of either hydrocarbon to covalently bound products was not observed in any of the incubations.

To determine whether naphthalene was metabolized to 1-naphthol and/or 1,2-dihydroxy-1,2-dihydronaphthalane, the methanol/water supernatant was concentrated to approximately half the original volume and measured. A 100 µl aliquot of the methanol/water phase was chromatographed on a 5 µ $C_{18}$ radial pak column (0.8 x 10cm) in 40% methanol/60% water for 10 min at 1 mL/min followed by a linear program to 70% methanol/30% water over 10 min. Naphthalene dihydrodiol eluted at 7 min and contained 15 and 72% of the total metabolite-associated radioactivity in lung and liver microsome incubations, respectively. Radioactivity coeluting with 1-naphthol (21 min) contained 71 and 21% of the total cpm eluting from the reverse phase column in extracts from lung and liver microsomal incubations. The data indicated that total dihydrodiol plus 1-naphthol formation in liver microsome incubations occurs at only 60% of the rate of formation of these two metabolites in lung microsomes while, consistent with earlier studies, kidney microsomes metabolized naphthalene very slowly. While NADPH-dependent metabolism of naphthalene occurred at easily measurable rates, no arachidonic acid-supported metabolism of naphthalene to naphthalene dihydrodiol or to 1-naphthol was detected in microsomal incubations prepared from lung, liver, or kidney.

TABLE 25

NADPH- and Arachidonate-Dependent Metabolism of Naphthalene or 2-Methylnaphthalene to Covalently Bound Products

| MICROSOMES | SUBSTRATE | COFACTOR | INHIBITOR | COVALENT BINDING[a] |
|---|---|---|---|---|
| Lung | NA | NADPH | None | 7.41 |
| Lung | NA | ARA | None | 0.07 |
| Lung | NA | ARA | Indo | 0.06 |
| Lung | NA | None | None | 0.13 |
| Liver | NA | NADPH | None | 5.77 |
| Liver | NA | ARA | None | 0.53 |
| Liver | NA | ARA | Indo | 0.39 |
| Liver | NA | None | None | 1.39 |
| Kidney | NA | NADPH | None | 0.03 |
| Kidney | NA | ARA | None | 0.03 |
| Kidney | NA | ARA | Indo | 0.01 |
| Kidney | NA | None | None | 0.03 |
| Lung | 2MN | NADPH | None | 1.56 |
| Lung | 2MN | ARA | None | 0.13 |
| Lung | 2MN | ARA | Indo | 0.15 |
| Lung | 2MN | None | None | 0.14 |
| Liver | 2MN | NADPH | None | 1.49 |
| Liver | 2MN | ARA | None | 0.20 |
| Liver | 2MN | ARA | Indo | 0.18 |
| Liver | 2MN | None | None | 0.16 |
| Kidney | 2MN | NADPH | None | 0.17 |
| Kidney | 2MN | ARA | None | 0.15 |
| Kidney | 2MN | ARA | Indo | 0.15 |
| Kidney | 2MN | None | None | 0.14 |

[a]Incubations were prepared in duplicate on ice in a total volume of 2 mL. Incubations contained: 2 mg microsomal protein, 0.1 mM $^{14}$C-naphthalene (6117 dpm/nmole) or 0.1 mM $^{14}$C-2-methylnaphthalene (23,560 dpm/nmole, added in 5 μ L methanol), NADPH-generating system, indomethacin (100 μ M, added in 5 μ L ethanol) and arachidonic acid (Sigma, 100 μ M, added in 5 μ l methanol, prepared fresh immediately before the incubation) as specified. Microsomes and indomethacin (indicated flasks) were added and the incubation bottles were transferred to a shaking incubator for 3 min at 37° C. The incubations were cooled to ice temperature, substrate, NADPH-generating system, and arachodonic acid were added as appropriate and the vessels were incubated for 6 min at 37°C. Values are reported as nmoles/mg microsomal protein/6 min. Abbreviations are: NA-naphthalene, 2MN-2-methylnaphthalene, ARA-arachidonic acid, and Indo-indomethacin.

## SUMMARY AND CONCLUSIONS

Intraperitoneal administration of either naphthalene or 2-methylnaphthalene to mice results in a highly selective dose-dependent lesion of the nonciliated cells of the pulmonary bronchiolar epithelium (Mahvi et al., 1977; Tong et al., 1982; Griffin et al., 1981; Warren et al., 1982). The fact that the Clara cell is a predominant target cell for naphthalene and that cytochrome P450 monooxygenases are thought to be highly localized in this cell type (Boyd, 1977; Serabjit-Singh et al., 1980; Devereux et al., 1981) suggested that the cytochrome P450-dependent metabolism of these aromatic hydrocarbons may play an important role in initiating the pulmonary bronchiolar lesion. Indeed, subsequent studies with naphthalene demonstrated that a reactive metabolite(s) from this compound becomes covalently bound to macromolecules in the lung in a dose-and time-dependent manner and that pretreatment with piperonyl butoxide or with diethyl maleate alleviates or exacerbates, respectively, the bronchiolar necrosis and covalent binding (Warren et al., 1982). However, at all doses and times after naphthalene administration, covalent binding to nontarget tissue macromolecules was higher than in target tissue. This, in conjunction with the data showing that naphthalene is metabolized to a number of chemically reactive metabolites (Horning et al., 1980; Stillwell et al., 1982) which can potentially combine irreversibly with macromolecules, and studies indicating that a reactive metabolite(s) formed in the liver appears to be capable of circulating and binding in the lung (Buckpitt and Warren, 1983), suggested that measurements of in vivo covalent binding may only provide a general indicator of the metabolism of naphthalene via "toxic" pathways. Since glutathione had been shown to modulate both the pulmonary toxicity of naphthalene in vivo (Warren et al., 1982) and covalent binding by naphthalene metabolites in vivo and in vitro (Warren et al., 1982; Smart and Buckpitt, 1983) and because numerous studies have shown that trapping reactive metabolites with glutathione followed by analysis of the resulting adducts can provide a useful means of monitoring the nature and quantities of reactive metabolite formed (Chasseaud, 1979), an HPLC method was developed which was capable of separating the three glutathione adduct peaks formed during the microsomal metabolism of naphthalene. Preliminary studies indicated that there were marked differences in the relative rates of formation of the three naphthalene glutathione adducts in incubations of target and nontarget tissue microsomes. Further studies to more fully characterize these differences indicated that the preferential formation of glutathione adduct 2 by mouse lung microsome incubations was due to the regio- or stereo-selectivity of naphthalene metabolism by cytochrome P450 monooxygenases or epoxide hydrolases but not by the glutathione transferases.

One of the major objectives of the studies conducted during the past contract year has been to provide structural identification of the glutathione adducts. While none of the glutathione adducts has been definitively identified, current evidence is consistent with the fact that these adducts are positional and enantiomeric derivatives of dihydroxydihydro-glutathionyl naphthalene. This evidence includes studies showing that all three adducts are formed during the chemical reaction of naphthalene-1,2-oxide with reduced glutathione at basic pH or at neutral pH in the presence of the glutathione transferases. In addition, negative surface ionization mass spectrometry of naphthalene glutathione adducts 1 and 2 yielded parent ions at M/Z 450, M/Z 432 (loss of 18, which is most likely due to dehydration of the aromatic nucleus) and M/Z 306 which is a fragment characteristic of glutathione adducts (Nelson et al., 1981; Moss et al., 1983). Sufficient quantities of both glutathione adduct 1 and 2 are available in pure form to do both proton and $^{13}$C-NMR. NMR spectra are currently scheduled on the 500 MHz NMR spectrometer at the University of California, Davis, CA and will be taken and interpreted by Professor Neal Castagnoli, Jr., Department of Medicinal Chemistry, University of California, San Francisco. $^{13}$C-NMR will be taken and interpreted by Drs. Castagnoli and Sidney Nelson (Professor of Medicinal Chemistry, University of Washington, Seattle). Small quantities of adduct 3 have been purified and are awaiting analysis by mass spectrometry.

Studies by Boyd and his coworkers (Dutcher and Boyd, 1979; Boyd et al. ,1978; Wolf et al., 1982) have shown that the target organ specificity for damage by the bronchiolar alkylating agent and cytotoxicant, 4-ipomeanol, does not correlate with the rate of microsomal metabolism of this agent to reactive metabolites at saturating substrate concentrations in target vs. nontarget tissues. Complete kinetic studies, on the other hand, demonstrated a good correlation between the target tissue specificity for damage by this furanoterpinoid and the $K_m$ for the formation of reactive metabolites. Thus, during the past contract year, the kinetics of formation of reactive naphthalene metabolites trapped as glutathione adducts have been investigated in detail. The formation of glutathione adducts from naphthalene is a two step process and depends upon both the cytochrome P450-dependent metabolism of the parent hydrocarbon and on the glutathione transferase-mediated conjugation. Therefore, incubation conditions had to be established so that the microsomal metabolic activation of naphthalene was the rate determining step in the formation of glutathione adducts. Studies reported previously (Buckpitt et al., 1984; Buckpitt, 1983) had identified the region of linearity for the formation of naphthalene glutathione adducts with time and microsomal protein in incubations of mouse lung and liver microsomes and had demonstrated that the use of 10 CDNB units of glutathione transferase activity was sufficient to promote the formation of conjugates at maximal rates.

Studies conducted during the past contract year indicated that the rate of formation of naphthalene dihydrodiol and all three glutathione adducts was linear with time in rat lung microsomal incubations for at least 12 min and with microsomal protein to at least 2 mg per incubation. Likewise, studies with hamster lung microsomes indicated that the formation of polar naphthalene metabolites was linear with time for 13 min and with protein concentrations to 2 mg/incubation. Thus, in all the kinetic studies reported, incubations were conducted for 6 min with 1 mg microsomal protein.

The estimated $K_m$ for the formation of each of the naphthalene glutathione adduct metabolites, which ranged from 2 to 96 μ M, must be viewed with caution since the lowest substrate concentration used in these kinetic studies was 30 μ M. Nevertheless, the data in Figure 7 do indicate that the $K_m$ for naphthalene glutathione adduct 2 formation in mouse liver and lung microsomes is very low, and that at environmentally realistic concentrations, the microsomal enzymes in both tissues are capable of rapidly metabolizing this aromatic hydrocarbon. The data also appear to show that there were not marked differences in the $K_m$ for the formation of any one of the metabolites in lung vs. liver microsomal incubations but that there were differences in the $K_m$ for the formation of conjugate 2 vs conjugates 1 and 3 in both lung and liver. The $K_m$ for conjugate 2 formation in lung and liver microsomal incubations was 2 and 9 μ M, respectively, while the $K_m$ for metabolism of naphthalene to adducts 1 and 3 was approximately 70-100 μ M. These data are consistent with the data shown in Table 7 which indicate that at low substrate concentrations the ratio of formation of adduct 2 to adducts 1 or 3 increases rather markedly. Indeed, at the lowest substrate concentration studied (31.2 μ M) the ratio of adduct 2 to 1 in lung microsomal incubations was 80 to 1 while at high substrate concentrations (1 mM), the ratio of adducts was 20-25 to 1. In contrast to the $K_m$ values where major differences were not noted between mouse lung and liver microsomal incubations, there were significant differences in the $V_{max}$ for metabolism of naphthalene to the individual adducts. Consistent with all of the previous studies which had demonstrated the preferential formation of adduct 2 in mouse lung microsomal incubations, the kinetic studies showed that the $V_{max}$ for formation of adduct 2 was approximately 20 times that for adducts 1 or 3 in lung and was about twice that of adduct 2 in mouse liver microsomal incubations.

Differences in the calculated $K_m$ values for the formation on adduct 1 and 3 vs adduct 2 in mouse lung microsomal incubations also were apparent in the comparative kinetic studies in lungs from sensitive vs nonsensitive species (Table 9). The $K_m$ for the formation of all three glutathione adducts was very low in both rat and hamster lung microsomal incubations and was similar to the calculated $K_m$ for formation of adduct 2 in mouse lung microsomal incubations. In

contrast to the slight difference noted in the $K_m$ for sensitive and nonsensitive species, there were marked differences in the maximal rate at which adduct 2 could be produced. At saturating substrate concentrations, mouse lung microsomes metabolized naphthalene to glutathione conjugate 2 at a rate of 14 nmoles/microsomal protein compared to 1-1.5 nmoles/min/mg in rat or hamster lung microsomal incubations (Table 8, Figures 6 and 7). These kinetic data are consistent with the fact that total polar metabolite formation in mouse lung microsomal incubations occur at a much higher rate than in hamster or rat lung microsomal incubations at high substrate concentrations. At low substrate concentrations, the rates are similar.

Studies on mouse liver and lung microsomal metabolism of naphthalene at varying concentrations of glutathione indicated that, as expected, the rates of formation of 1-naphthol, naphthalene dihydrodiol and covalently bound metabolites were inversely related to the concentration of glutathione in the incubations. Conjugate formation was at or near maximal at 2.5-5.0 mM glutathione concentrations and at this glutathione concentration, the formation of other oxygenated metabolites occurred at approximately 15% of the rate of total glutathione adduct formation. Plots of the rate of 1-naphthol + dihydrodiol vs covalently bound metabolite formation at different concentrations of glutathione were consistent with, but did not preferentially support, the view that at least a portion of the reactive metabolites which become bound covalently from naphthalene do not arise from the intermediate formation of 1-naphthol.

In marked contast to studies by others (Prough et al., 1979; Lorenz et al., 1979; Oesch et al., 1980; Jakobssen et al., 1982) who have reported that the cytochrome P450-dependent metabolism of a number of substrates occurs at a rate which is either undetectable or is at the limits of assay sensitivity, studies conducted during the past contract year were able to demonstrate easily measureable rates of naphthalene metabolism by human lung microsomal enzymes. Consistent with the studies by Oesch et al. (1980) who demonstrated very high levels of epoxide hydrolase activity in human lung, the predominant pathway for naphthalene metabolism in human lung microsomal incubations was to the dihydrodiol. Marked interindividual differences were noted in the rates of naphthalene metabolism. Microsomes from 1 of the 3 tissue samples obtained were capable of causing a substantial decrease in mouse liver microsome-catalyzed formation of polar naphthalene metabolites and thus additional studies are needed to determine whether an "inhibitor" is present in human lung microsomal fractions and, if so, to provide further characterization of this inhibitor. Additional studies are also needed to more completely characterize the kinetics of human lung microsomal metabolism of naphthalene.

Recent data from several laboratories have suggested that the formation of covalently bound metabolites from a number of aromatic hydrocarbons including benzene (Tunek et al., 1980; Sawahata and Neal, 1983; Lunte and Kissinger, 1983), bromobenzene (Hesse et al., 1980) and naphthalene (Hesse and Metzger, 1979; Schwartz et al., 1980) involves the intermediate formation of a monohydroxylated derivative. While it appears that covalently bound metabolites from benzene arise solely through phenol, this does not appear to be the case with either bromobenzene (Lau et al., 1984; Monks et al., 1982) or naphthalene (Buckpitt et al., 1984). Evidence which does not support the view that all of the covalently bound metabolites from naphthalene arise through 1-naphthol as an intermediate includes the demonstration that mouse lung microsomes metabolize naphthalene to 1-naphthol at approximately 2.5 times the rate of mouse liver microsomes yet the rate of formation of covalently bound metabolites from naphthalene is similar. The difference is not due to lower rates of formation of covalently bound metabolites from 1-naphthol in mouse lung vs liver microsomes; microsomes from these two tissues catalyze the formation of covalently bound metabolites from 1-naphthol at very similar rates. Furthermore, addition of reduced glutathione to mouse hepatic microsomal incubations containing $^{14}$C-1-naphthol and cytosol (containing the glutathione transferases) did not result in the formation of any of the adducts which are separated and quantitated by HPLC. Previous in vivo studies have shown that the levels of radioactivity covalently bound to tissue macromolecules after i.p. administration of 1-naphthol are similar to those observed after

administration of comparable doses of naphthalene (Buckpitt, 1983). Naphthalene-1,2-oxide [$^3$H] has been prepared synthetically and will be used in an upcoming experiment to determine whether tritium is bound covalently to protein in microsomal incubations done in the presence or absence of NADPH or glutathione.

As indicated earlier, previous in vivo studies have provided evidence in support of the view that reactive naphthalene metabolites produced by hepatic cytochrome P450 monooxygenases are capable of leaving their site of origin and becoming bound covalently to macromolecules in other organs (Buckpitt and Warren, 1983). Such metabolites could serve to modulate the toxicity of naphthalene in two ways: 1, by compromising the defense mechanisms (i.e. the glutathione conjugation system) in extrahepatic target organs and/or 2, by interacting directly with bronchiolar cells to result in the injury observed after administration of naphthalene. Several experiments have been conducted in the past contract year in an attempt to establish the quantitative role of reactive naphthalene metabolites produced in the liver in the bronchiolar injury.

To quantitatively determine naphthalene oxide effluxing from intact liver cells, hepatocytes were prepared from mouse liver by collagenase perfusion and brought to> 96% viability (as assessed by trypan blue exclusion) by Percoll density gradient centrifugation. Since glutathione will not penetrate intact hepatocytes, $^3$H-glutathione was added along with semipurified glutathione transferases to hepatocyte incubations with naphthalene. The results of initial experiments have indicated that> 75% of naphthalene oxide formed intracellularly is capable of becoming conjugated with glutathione extracellularly. This appears to be the case at both 0.5mM and 0.05 mM naphthalene concentrations which would suggest that intracellular stores of glutathione do not necessarily have to be depleted before naphthalene oxide leaves the hepatocyte. The mechanism for formation of a polar, reasonably reactive molecule on the endoplasmic reticulum and its ability to reach the exterior of the cell without undergoing nucleophilic attack by glutathione will be examined in detail in future investigations. It is possible that, once formed, naphthalene oxide travels down the interior portion of the endoplasmic reticulum and is therefore protected from conjugation and hydrolysis. Whether naphthalene oxide is extruded from the cell in a lipid/lipoprotein envelope is also an important question which will be the subject of future research.

Relatively high doses of naphthalene oxide administered intravenously were required to achieve significant depletion of pulmonary glutathione (Figure 12). It is unlikely that, even after administration of large doses of naphthalene, similar levels could be achieved through formation and efflux of this oxirane metabolite from the liver. It is possible that if naphthalene oxide arising from metabolism of the parent hydrocarbon in the liver is protected from nucleophilic attack by water or by thiols in the blood stream (and there is some evidence to support this), that it will be biologically far more active than naphthalene oxide administered in ethanol via the tail vein. If upcoming experiments indicate that the half life of naphthalene oxide generated by hepatocytes is different from chemically prepared material added to incubation medium, then attempts will be made to encapsulate naphthalene oxide in liposomes. It will also be important in upcoming studies, designed to examine the pulmonary toxicity of several metabolites of naphthalene administered intravenously, to determine whether the pulmonary capillary endothelial cell is a potential target cell for metabolites generated in the liver. There is ample precedent for such a possibility from studies done with pyrrolizidine alkaloid metabolites (Mattocks and Driver, 1983).

Finally, a series of studies designed to explore the possibility that cobalt protoporphyrin could be used to differentially block hepatic without affecting pulmonary monooxygenase activity has been completed. Such a compound could be an important tool in examining the relative importance of extrahepatic vs hepatic metabolism in organ toxicities of the lung or kidney associated with the metabolic activation of a number of environmental and industrial

cytotoxicants. Administration of cobalt protoporphyrin either subcutaneously or intravenously resulted in a marked and sustained decrease in both hepatic and pulmonary microsomal metabolism of naphthalene to covalently bound metabolites and in no apparent protection from naphthalene or 2-methylnaphthalene-induced bronchiolar damage in vivo. While the exact mechanism for this lack of inhibition of naphthalene-induced bronchiolar injury is unclear, it may be related to differences in the distribution of naphthalene in the vehicle vs the cobalt protoporphyrin treated animal. Cobalt protoporphyrin pretreatment was shown to cause a marked and sustained decrease in hepatic glutathione levels and since this is a major metabolic pathway for naphthalene in mouse liver, it may result in a marked increase in the amount of unchanged hydrocarbon reaching the lungs. These studies illustrate the care with which in vivo studies using P450 inhibitors to examine a role for metabolism in the cytotoxicity of a given agent must be interpreted. They also indicate that cobalt protoporphyrin may not be an appropriate agent to test the role of metabolism in pulmonary toxicities by chemicals in vivo.

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